Emerging Technologies for the Assessment of Bovine Immunoglobulins in Biofluids

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ABSTRACT

Measurement of bovine serum and colostrum immunoglobulin G (IgG) concentration is critical for colostrum and calf health monitoring in order to determine the colostrum quality and failure of transfer of passive immunity (FTPI), which is considered the main reason for increased morbidity and mortality rates in newborn calves. Several qualitative and quantitative assays are available, but radial immunodiffusion assay is acknowledged as the reference method. However, it is expensive and takes a long time to obtain the results, which prevents the identification of calves with FTPI prior to gut closure. As a consequence, there is a demand for rapid, accurate and inexpensive diagnostic assays for IgG. The objectives of this thesis were: (1) to develop and validate an infrared (IR) spectroscopy based assay for quantification of bovine serum and colostrum IgG concentration, and (2) to validate a novel and rapid on-farm tool for detection of FTPI in dairy calves and for assessing colostrum quality of dairy cows.

Infrared spectroscopy has recently emerged as a powerful, reagent-free diagnostic tool for the quantitative characterization of biological fluids in human and veterinary medicine. We set out to develop a quantitative assay based on IR spectroscopy to measure bovine serum and colostrum IgG concentration and to compare the values with that of the RID assay. Although there are a number of IR spectroscopic sampling techniques, in this research, transmission-IR and attenuated total reflectance (ATR-IR) spectroscopy were used. For quantification of bovine serum IgG concentration, transmission-IR and ATR-IR assays were developed using 250 serum samples collected from calves. The IgG concentration measured by both assays showed excellent

correlation with RID-measured IgG. Also, both IR-based assays showed potential for detection of FTPI with good to excellent sensitivity, specificity and accuracy. The transmission-IR assay showed slightly higher precision than the ATR-IR assay. However, the ATR-IR assay is more appropriate for farm and veterinary clinic use. For quantification of colostral IgG concentration, a transmission-IR assay was developed using 251 colostrum samples. The IgG measured by the IR assay showed excellent levels of agreement with the RID assay. The results suggest that IR spectroscopy may be a useful method for colostrum monitoring programs.

Evaluation of an initial version of ZAPvet Bovine IgG test for detection of calves with and without FTPI revealed that the ZAPvet test is relatively sensitive and would be acceptable as an initial screening test for diagnosis of FTPI in dairy calves. However, the low specificity of ZAPvet test would result in over prediction of FTPI incidence, which could result in unnecessary interventions for calves with adequate transfer of passive immunity.

Validation of refractometers, either digital Brix (Atago Co. Ltd; WA) or optical STP (Westover RHC-200ATC handheld refractometer, Woodinville, WA), for detection of FTPI in 202 dairy calves, and digital Brix (Atago Co. Ltd; WA) or optical Brix (model 300001; SPER Scientific, Scottsdale, AZ), for assessing of quality of 251 colostrum samples, revealed that the digital and optical refractometers have good potential for being a useful and practical on-farm management tools to be included in colostrum and calf health monitoring program on dairy operations. Furthermore, the two refractometers performed similarly for detection of FTPI in dairy calves and for assessing of colostrum quality of dairy cows with cut-points slightly higher than that reported in recent studies.

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DEDICATION

To my wife and parents, for their love, support and encouragement

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LIST OF ABBREVIATIONS

ADG	average daily gain
ATPI	adequate transfer of passive immunity
ATR	attenuated total reflectance
CV	coefficient of variation
dL	deciliter
ELISA	enzyme-linked immunosorbent assay
FN	false negative
FP	false positive
FTPI	failure of transfer of passive immunity
GGT	γ-glutamyl transferase
Ig	Immunoglobulin
IgG	immunoglobulin G
IR	infrared
mg	milligram
NPV	negative predictive value
PLS	partial least squares
PPV	positive predictive value
r	Pearson correlation coefficients
RER	range error ratio
RID	radial immunodiffusion assay
RMMCCV	root mean squared error of the Monte Carlo cross-validation value
RMSEC	root mean squared error of calibration
RMSEP	root mean squared error of prediction
RPD	ratio of predictive deviation
SD	standard deviation
Se	sensitivity
SNV	standard normal variate
Sp	specificity
TIA	turbidimetric immunoassay
STP	serum total protein

CHAPTER 1. GENERAL INTRODUCTION

1.1 Failure of transfer of passive immunity

The bovine placental structures separate the maternal and fetal blood supplies (Godden, 2008). Therefore significant *in utero* transmission of immunoglobulins (Igs) from the dam to the fetus does not occur (Barrington and Parish, 2001). Consequently, newborn calves have no maternally supplied immunological defenses against the environmental pathogens at the time of parturition. They entirely depend on the intestinal absorption of maternal Igs through the consumption of colostrum (Baintner, 2007). Immunoglobulins are present in bovine colostrum in different concentrations, IgG_1 (80%), IgG_2 (5 to 10%), IgM (5%) and IgA (5 to 7%). As the most abundant Ig in colostrum, IgG occurs in higher serum levels in colostrum-fed calves (Stott et al., 1979; Larson et al., 1980). Calves that fail to ingest and absorb a sufficient amount of colostral IgG are defined as suffering from failure of transfer of passive immunity (FTPI). Calves are classified as having FTPI if their serum IgG level is less than 1000 mg/dL (Godden, 2008).

Calves with serum IgG greater than 1000 mg/dL have a significantly reduced risk of death compared to calves with lower serum IgG (Chelack et al., 1993). Similarly, calves with serum IgG levels between 890 to 1300 mg/dL were associated with increased risk of calf death (Virtala et al., 1999). Other studies, demonstrated that lower serum IgG value (500 mg/dL) is associated with higher relative risk of calf death (Rea et al., 1996; Donovan et al., 2008). Many studies suggest that transfer of passive immunity is adequate when serum IgG concentrations are a minimum of 1000 mg/dL. Thus, this value has been widely accepted as a cut-point for FTPI.

Technically FTPI is not a disease, but rather is a major risk factor associated with calf dehydration, diarrhea and dullness that leads to increased risk of neonatal morbidity and mortality (Tyler et al., 1996; Weaver et al., 2000). It has been estimated that ~ 39 to 50% of preweaned calf mortality in the United States is associated with FTPI (Margerison et al., 2005). Calves with FTPI show reduced average daily gains (ADG) in the first month of life, and body weight has been reported to be significantly reduced (by as much as 2.1 kg in one study) compared to calves without FTPI in first three months of life (Virtala et al., 1996). The effect of FTPI has been shown to extend beyond the neonatal period in heifer calves, affecting long-term productivity and resulting in decreased milk yields and increase culling rates during first lactation (DeNise et al., 1989; Heinrichs and Heinrichs, 2011).

It is recognized that FTPI is widespread on farms across North America. The reported FTPI prevalence in dairy calves ranges from 19 to 40% (Dairy, 2007) and in beef calves from 11 to 31% (Perino, 1997). In a recent study the reported prevalence in US dairy farms is 19.2% (Beam et al., 2009), in contrast to values of 37.1% and 19% for dairy farms in Ontario and Quebec respectively (Trotz-Williams et al., 2008; Filteau et al., 2003). More recent studies have reported that 11% of calves from farms in Ontario and Minnesota have FTPI (Windeyer et al. 2012), compared with a reported prevalence of FTPI in Alberta dairy farms is 27.8% (Bartier et al., 2015). To date, there is no published information about the prevalence of FTPI in Atlantic Canada dairy farms. As previously mentioned, FTPI is a result of reduced ingestion or absorption of colostrum (McGuirk and Collins, 2004). Therefore, the prevention of FTPI is associated with proper colostrum and calf management practices, which will be discussed next in this review.

1.2 Factor influencing failure of transfer of passive immunity

Several factors are reported to have an effect on transfer of passive immunity in calves including, colostrum quality, efficiency of colostral Ig absorption, time of colostrum feeding, and the volume and method of colostrum administration.

1.2.1 Colostral IgG quality

Colostrum contains high concentrations of nutritional components, growth factors and immunoglobulins (IgG, IgA, IgM, and IgE) (Kehoe et al., 2007; Bielmann et al., 2010). IgG represents 85-90% of total Igs in colostrum, with 80-90% of this being IgG_1 (Larson et al., 1980; Baumrucker et al., 2010). The IgG concentration in colostrum has traditionally been considered the hallmark for evaluating colostrum quality (Godden et al., 2009). In dairy cows, good quality colostrum is widely considered to contain IgG concentration greater than 50 g/L (McGuirk and Collins, 2004). Colostral quality varies widely within and between farms. The reported range of colostral IgG at the farm level has been reported at 7.1 to 159 g/L (Quigley et al., 2013), which is similar to the range reported in recent studies (Bartier et al., 2015; Morrill et al., 2015). The proportion of poor quality colostrum (IgG <50 g/L) has been described as ranging from 16 to 32% (Chigerwe et al., 2008; Morrill et al., 2012; Quigley et al., 2013). Therefore, due to the variation in colostral IgG concentration, it is important to assess colostrum quality before feeding to calves to ensure these neonates will receive adequate amounts of IgG.

1.2.2 Efficiency of colostrum Ig absorption

During the first 24 to 36 hours of life, bovine neonatal enterocytes have the ability to absorb non-selectively a variety of macromolecules by pinocytosis, including Ig (Broughton and Lecce, 1970). In this process, the Ig molecules are transported across the cells and released into the lymphatics by exocytosis, then gain access to the general circulation through a thoracic duct (Staley et al., 1972). The absorption of colostral Ig gradually decreases after birth with complete cessation of gut non-selective absorption at approximately 24-36 hours postpartum. This coincides with the maturation of gut cells and other changes such as decreases in abomasal pH and the initiation of the intestinal secretion of digestive enzymes (Quigley et al., 2002). The efficiency of colostral Ig absorption is affected by time, volume and method of colostrum feeding.

1.2.2.1 Time of colostrum feeding

The age of the calf at the initiation of colostrum feeding is one of the major factors affecting the efficiency of Ig absorption. The absorption of colostral Ig linearly decreases with the time from birth to gut closure (Weaver et al., 2000). Gut closure occurs approximately at 24 hours postpartum and may be extended to 36 hours if colostrum feeding was delayed (Stott et al., 1979). The optimal absorption of IgG occurs within 4 hours after birth and declines rapidly after 6 hours. Calves fed colostrum more than 4 hours after birth are 2.7 times more likely to have FTPI than calves fed colostrum within 4 hours (Beam et al., 2009). Furthermore, for every 30-minute delay in colostrum consumption, serum IgG concentrations decrease by 200 mg/dL (Rajala and Castrén, 1995).

1.2.2.2 Volume of colostrum feeding

The minimum amount of IgG that should be consumed is 100 g (Davis and Drackley, 1998) while others suggest that 150 to 200 g IgG is more appropriate (Chigerwe et al., 2008). Several studies have shown that calves fed a large volume (4 L) of colostrum had significantly higher serum total protein (STP) levels than calves fed 2 L when fed by the same method at the same time after birth (Morin et al., 1997; Godden et al., 2009). To ensure adequate transfer of passive immunity, calves should be fed between 2 and 4 L of colostrum depending on the calf's body weight and the IgG concentration in the colostrum (McGuirk and Collins, 2004; Kaske et al., 2005). In a single feeding, Holstein calves may consume 4 or more liters of colostrum and at 12 hours of age can also consume an additional 3 L (Chigerwe et al., 2009). This suggests that calves can normally consume a high volume of colostrum if it is offered. Consequently, this will significantly reduce the risk of developing FTPI (Chigerwe et al., 2009).

1.2.2.3 Method of colostrum administration

Ontario dairy farms that leave dairy calves more than three hours with the dam to nurse have a significantly higher risk of calves with FTPI compared to farms that reported not leaving a calf to suckle their dam (Trotz-Williams et al., 2008). A study showed that 25% of US dairy farms allow calves to suckle the dam to obtain colostrum, and 75% of dairy farms are hand-fed, with 89% of those calves being bottle feed and the remaining fed via oesophageal tube (Beam et al., 2009). In agreement with the findings of the Ontario study (Trotz-Williams et al., 2008), the same study indicated that 61.4% of

calves that received colostrum via suckling dam developed FTPI compared to 19.3% that were bottle-fed and 10.8% that were tube-fed (Besser et al., 1991). On a subsection of calves, feed a small colostrum volume, calves fed the same volume of colostrum had higher IgG levels when fed by bottle than by tube (Godden et al. 2009a). Bottle-feeding is considered to increase passive transfer due to the closure of the esophageal groove, compared to tube-feeding that does not allow this closure (Godden et al., 2009b). Another study showed that calves fed via esophageal tube had higher STP concentrations compared to bottle-fed calves. However, the calves in this study were not fed the same volume; tube-fed calves received 4 L, and bottle-fed calves received 2 L (Kaske et al. 2005). A recent study by Chigerwe et al., (2012) reported that the methods of colostrum feeding has no effect on transfer of passive immunity when calves feed the same volume of colostrum.

1.3 Factors influencing colostrum quality

1.3.1 Breed

Cow breed may affect colostrum quality due to genetic differences and dilution factors (Godden, 2008). The colostrum of Jersey cows has the highest level of total Ig (9%) because of lower volume of colostrum produced. Comparatively, Holstein cows produce colostrum with the lowest concentration of total Ig (5.6%), followed by Brown Swiss (6.6%), and Ayrshire (8.1%) (Muller and Ellinger, 1981).

1.3.2 Age of dam

Most of the studies have reported that older cows produce better quality colostrum. However, Muller and Ellinger, (1981) found no statistical difference in colostral Ig concentrations in Holstein, Jersey and Guernsey cows in their 1st, 2nd and 4th lactation or greater. However, cows in their 3rd lactation showed significantly higher Ig concentrations. Similarly, Tyler et al., (1999) confirmed that Holstein and Guernsey cows in their 3rd or greater lactation have significantly higher immunoglobulin concentrations than cows in 1st and 2nd lactation. The authors also reported no significant difference existed between colostral IgG concentrations and lactation number in the 1st and 2nd lactations.

1.3.3 Dry period length

The secretion of Ig from dam circulation to mammary gland begins approximately five weeks before calving (Godden, 2008). Therefore, cows with a dry period less than five weeks are more at risk of producing low-quality colostrum (Dixon et al., 1961; Rastani et al., 2005). Holstein cows need at least a 3-4 week dry period to produce good quality colostrum. When the dry period is short (40 days), cows produce 2.2 kg colostrum less than cows with a longer dry period (60 days) (Staley et al., 1972).

1.3.4 Volume of colostrum produced

The volume of colostrum produced is one of the factors that affects colostral IgG concentration. Morin et al., (2010) has reported that for every liter of colostrum produced by Holstein-Friesian cows, the colostral IgG concentration decreases by 3.7%. This concurs with an earlier report by Pritchett et al., (1991) that observed Holstein-Friesian

cows are more likely to produce high quality colostrum if the volume of colostrum produced at first milking is less than 8.5 kg. However, a more recent study by Grusenmeyer et al., (2006) reported that there is no predictable relationship between colostrum IgG concentration and weight of colostrum produced at first milking.

1.3.5 Delayed colostrum collection

The quality of colostrum decreases the longer it is retained in the udder. A study by Moore et al., (2005) showed that delaying colostrum harvesting for 6, 10 or 14 hours after calving resulted in decreases of colostral IgG concentration of 17%, 27%, and 33%, respectively, relative to a 2 hour baseline. Milking cows within 1 to 2 hours after calving increases the chance to collect colostrum with high quality (Godden et al., 2008).

1.4 Current methods for measuring IgG concentration

Several diagnostic tests are available for measuring serum and colostral IgG concentrations either directly or indirectly. Direct methods include radial immunodiffusion (RID) assay, enzyme-linked immunosorbent assay (ELISA) and an automated turbidimeteric immunoassay, while indirect methods include refractometry, colostrometer (colostral IgG only), the sodium sulfite turbidity test, zinc sulfate ($ZnSO_4$) turbidity test, whole blood glutaraldehyde coagulation test and serum γ-glutamyl transferase (GGT) activity (Weaver et al., 2000).

1.4.1 Direct methods

1.4.1.1 Radial immunodiffusion (RID) assay

Radial immunodiffusion (RID) assay has been used successfully to directly and quantitatively measure bovine serum and colostrum IgG concentrations (Weaver et al., 2000; Godden, 2008). This assay uses an anti-IgG antibody dissolved in an agarose gel. Briefly, the sample is pipetted into small circular wells punched in the gel and left to precipitate. The diameter of the rings formed by the precipitation of the antigen/antibody complex is measured and compared to the size of rings formed by known concentrations of IgG. Diameters of reference sera precipitating ring are used to form a standard curve against which the IgG concentration of an unknown sample is determined (McBeath et al., 1971).

Although, RID has long been considered the current gold standard for measuring serum and colostral IgG concentration (McBeath et al., 1971), it is a laboratory-based assay that requires trained laboratory technicians, is not amenable to automation, utilizes reagents, is more expensive than the alternatives (Riley et al., 2007; Bielmann et al., 2010) and needs a long incubation time (18 - 24 hours) to obtain the results. These limitations impede the identification of calves with FTPI prior to gut closure. Although considered the gold standard, variability in the RID assay replicates from the same sample has been noted and is attributed to inaccuracies associated with the internal standards or operator error (Ameri and Wilkerson, 2008).

1.4.1.2 Enzyme-linked immunosorbent (ELISA) assay

Recently, various quantitative and semiquantitative enzyme-linked immunosorbent (ELISA) assays have become commercially available for the detection of

IgG concentrations in colostrum, milk and plasma (Vetter et al., 2013; Baumrucker and Bruckmaier, 2014; Gelsinger et al., 2015). A recent study reported a weak correlation between IgG concentrations determined by ELISA and RID assay in plasma (r = 0.59) and colostrum (r = 0.36). Moreover, the IgG concentration measured by ELISA was significantly lower than that measured by RID assay (Gelsinger et al., 2015). Another study found that there is no systematic bias between serum IgG concentration measured by RID and ELISA with mean of the difference between the two assays was 140 mg/dL (Lee et al., 2008). The Se (98%) of the ELISA assay to determine calves with FTPI has been shown to be higher than all the indirect methods while the Sp (91%) was lower than the indirect methods except refractometer, when compared to the reference RID assay (Lee et al., 2008).

1.4.1.3 Turbidimetric immunoassay (TIA)

The turbidimetric immunoassay (TIA) is a rapid, accurate and precise analytic method that provides a quantitative measurement for bovine serum and plasma IgG concentrations in a laboratory setting (Etzel et al., 1997). TIA measurements have been positively correlated (r = 0.99) with the RID method. However, the TIA method has been shown to have benefits over the RID assay including complete automation and greatly decreased time to obtain the results (Etzel et al., 1997; Davis et al., 2005). The only limiting factor of this assay reported was the relatively high cost per test, and its unsuitability for use in the field. Recently, a portable, quantitative, automated TIA was used for measurement of bovine IgG concentration and detection of FTPI in dairy calves under field conditions (Alley et al., 2012). The automated TIA, used the same principles

described by Etzel et al., (1997) and was connected to a portable analyzer (MBC QTII; Midland BioProducts Corp.). The serum IgG concentrations measured by the automated TIA closely paralleled those obtained by RID assay, with a coefficient of determination value of 0.98 (Alley et al., 2012).

1.4.2 Indirect methods

1.4.2.1 Refractometer

Digital and optical refractometers have been used for indirect estimates of serum and colostrum IgG concentrations (Deelen et al., 2014; Bartier et al., 2015). Estimation of total protein concentrations in serum and colostrum by refractometers was first proposed by McBeath et al., (1971). Refractometry is a rapid and inexpensive method for diagnosis of FTPI in calves, and for the assessment of colostrum quality of dairy cows. Numerous reports in the literature have documented the relationship between serum total protein (STP) and IgG concentration. In a study of 185 calves, the STP measured by refractometer was shown to have good correlations (r = 0.72) with serum IgG as measured by RID assay (McBeath et al., 1971). For the diagnosis of FTPI in calves, an STP of ≥ 5.2 g/L is the most appropriate cut-off for determining ≥ 1000 mg/dL serum IgG level in clinically normal calves (Calloway et al., 2002). However, in clinically ill newborn calves, an STP cut-off of ≥ 5.5 g/L is more appropriate to indicate the adequate transfer of passive immunity (Tyler et al., 1999).

Originally developed to measure the amount of sugar dissolved in water as a percentage of weight, the Brix refractometer has recently been recognized as a tool to determine bovine serum and colostrum IgG concentration (Bielmann et al., 2010; Morrill

et al., 2012; Quigley et al., 2013). A Brix score of < 7.8% Brix may be used to identify calves with FTPI, which results in a sensitivity and specificity of 90% and 94%, respectively (Morrill et al., 2013).

A value of 22% Brix has been equated to a colostral IgG concentration of 50 g/L and identified as an appropriate cut-point for both digital and optical Brix refractometers to differentiate between poor and good quality colostrum (Chigerwe et al., 2008; Bielmann et al., 2010). Various studies have reported the correlation between Brix scores and colostral IgG concentrations measured by RID assay, ranging from 0.64 to 0.94 (Bielmann et al., 2010; Morrill et al., 2012; Quigley et al., 2013). It has been used at different cut-points (ranging from 20 to 23% Brix) for the assessment of colostrum quality (Chigerwe et al., 2008). Of these, the best combination of sensitivity (65.7%) and specificity (82.8%) for detection of poor quality colostrum has been reported at a cut-point of 23% Brix (Bartier et al., 2015). Although the refractometer can be implemented as a rapid on-farm monitoring tool for colostrum management and as a predictor of calf health, only 2.1% of US farms have used this approach for routine monitoring according to the 2007 National Animal Health Monitoring System report (Dairy, 2007).

1.4.2.2 Colostrometer

The colostrometer is the most common tool used on-farm to assess colostrum quality (Dairy, 2007). Colostrometers are used to measure the specific gravity of colostrum that has been highly correlated with colostrum IgG content (Fleenor and Stott, 1980). Several studies have tested the colostrometer against RID measured IgG levels in order to determine its diagnostic test characteristics. Pritchett et al., (1994) used a cut-

point range of 60 to 85 mg/mL to reduce the number of false negative misclassifications. However, Chigerwe et al., (2008) found that the cut-point of 70 mg/mL is most consistent with 50 mg/mL IgG as measured by RID and resulted in Se and Sp of 75% and 78%, respectively.

A more recent study reported that the colostrometer data were correlated (r = 0.77) with RID results, and the highest combination of Se (84%) and Sp (77%) occurred at 80 mg/mL, compared with a cut-point of 50 mg/mL IgG as measured by RID (Bartier et al., 2015). Chigerwe et al. (2008) suggests that a cut-point of 70 mg/mL offers high sensitivity and specificity (75% and 78%, respectively) and recommends using a cut-point lies in the range of 60 to 90 mg/mL to reduce misclassification of false negatives. Other studies documented that colostrometer is not sufficiently sensitive or specific and works accurately only within a small temperature range (McGuirk and Collins, 2004). In addition to being affected by temperature (Mechor et al., 1992), the colostrometer is fragile (i.e. made of glass) (Bielmann et al., 2010), and its results are affected by non-IgG protein and the fat content of colostrum (Morin et al., 2001). Although the assessment of colostrum quality is critical, only 4% of farms in Quebec were reported to use the colostrometer consistently (Vasseur et al., 2010). In the US, 13% of farms assess the colostrum quality, with 43.7% of these farms using a colostrometer for routine monitoring according to the 2007 National Animal Health Monitoring System report (Dairy, 2007).

1.4.2.3 Sodium sulfite turbidity test

The sodium sulfite turbidity test is a 3-step semiquantitative test using 14, 16, and 18% solutions of sodium sulfite (Pfeiffer and McGuire, 1977). The measured endpoint of the test is the turbidity of the solutions that results from selective precipitation of high molecular weight proteins including immunoglobulins. The test solution with a higher sodium sulfite concentration will induce turbidity at lower concentrations of high molecular weight proteins. Consequently, turbidity resulting from the 14% test solution indicates a higher serum IgG concentration than turbidity resulting from 16 and 18% test solutions (Pfeiffer and McGuire, 1977). Using either the 14 or 16% test solution has been associated with a large number of calves with adequate serum IgG concentrations misclassified as having FTPI. The 18% test solution has been associated with optimal diagnostic utility in an examination of 242 calves, with both sensitivity and specificity equalling 85%. (Tyler et al., 1996). However the 14 and 16% test solution show high sensitivity (100%) and poor specificity (56 and 3%, respectively) for detection of calves with FTPI. The 18% test solution correctly classified a higher percentage (86%) of calves than the 14 % (71%) and 16% (37%) test solutions (Tyler et al., 1996). Therefore, the sodium sulfite turbidity test is best used as a single dilution procedure using the 18% test solution (Weaver et al., 2000).

1.4.2.4 Zinc sulfate turbidity test

The zinc sulfate turbidity test is a FTPI field screening test that is relatively cheap and rapid. The test operates on the same basic principle as the sodium sulfite turbidity test. However, it is typically performed as a single dilution assay in which 0.1 mL of

serum is added to 6 mL of 208 mg/L solution and then incubated for 30 minutes (Weaver et al., 2000). Using this dilution, the assay has demonstrated excellent sensitivity (100%), but very poor specificity (52%), and correctly classified 69% of calves tested (Tyler et al., 1996). The performance of the zinc sulfate turbidity test can be improved by increasing the concentration of the test solution. In one study, the concentration of the test solution was increased from 208 to 350 mg/L and then used to test 235 calves. The test sensitivity was decreased from 100 to 94% while specificity was dramatically increased from 25.5 to 76.5% (Hudgens et al., 1996). Although the zinc sulfate turbidity test is rapid and economic, it has major limitations including the effect of sample hemolysis and reagent quality on test results (Pfeiffer and McGuire, 1977; Hudgens et al., 1996; Tyler et al., 1996).

1.4.2.5 Whole blood glutaraldehyde coagulation test

A whole blood glutaraldehyde coagulation test has been used to estimate gamma globulins in cattle and the detection of FTPI in calves (Sandholm, 1974; Sandholm, 1976). The test can be performed under field conditions by adding 1.5 mL of whole blood to prepared glutaraldehyde solution and recording the time to clot formation. If the clot formation time is less than 5 minutes, this indicates the adequate transfer of passive immunity (Tyler et al., 1996).

The glutaraldehyde coagulation test was developed to address the challenges of turnaround time and economic constraints, but the test performance has been proven to be inadequate for routine diagnostic use with a sensitivity ranging from 0 to 41%, and specificity from 85 to 100% (Tennant et al., 1979; Tyler et al., 1996). Moreover, cross-

reactions with fibrinogen have been observed, and thus, calves with inflammatory diseases may be misclassified as having adequate transfer of passive immunity (Tennant et al., 1979).

1.4.2.6 Serum γ-glutamyl transferase (GGT) activity

γ-glutamyl transferase (GGT) is an enzyme synthesized in the mammary gland and is observed in calf serum after ingestion of colostrum (Perino et al., 1993). Although the association between serum GGT concentrations and serum IgG concentration is low (r = 0.41), Parish and colleagues suggested the prediction of FTPI in dairy calves on the basis of age and serum GGT (Parish et al., 1997). One, four, and seven-day-old dairy calves with serum GGT activities >200 IU/L, >100 IU/L, and >75 IU/L, respectively, have been classified as having adequate passive immunity transfer. However, dairy calves in their first two weeks of life with serum GGT activities <50 IU/L, have been found to be consistent with having FTPI (Parish et al., 1997). In contrast, others have reported that serum GGT activity was of little value in the prediction of FTPI in beef calves (Wilson et al., 1999). Serum GGT activity may be useful to confirm the ingestion of colostrum, but not for the assessment of serum IgG concentration. Therefore, serum GGT activity in calves is discouraged as a method for assessment of FTPI in calves, particularly beef calves (Weaver et al., 2000; Massimini et al., 2006).

1.5 Novel methods for measuring IgG concentration

1.5.1 Infrared spectroscopy

Commercially available since the 1940s, infrared (IR) spectroscopy is one of the most important tools in analytic chemistry (Günzler and Gremlich, 2002). One of the great advantages of IR spectroscopy is that virtually any sample, in virtually any state, can be tested. By the mid of the 1950s, a significant advancement in IR spectroscopy came with the innovation of Fourier transform infrared (FTIR) (Barth and Haris, 2009).

The use of FTIR in combination with the development of digital data acquisition has contributed to the enhancement of the accuracy and reproducibility of IR spectroscopy (Schultz, 2002; Dubois and Shaw, 2004). Today, IR spectrometer applications have been extended from analytic chemistry community to solve diagnostic problems in human and veterinary medicine.

1.5.1.1 Theory and principles of IR

Electromagnetic radiation is divided into frequency dependent regions including radiowave, microwave, infrared, visible, ultraviolet, x-rays and γ-rays (Stuart, 2005). The IR portion of the electromagnetic spectrum is divided into three regions, near (14285-4000 cm^{-1}), mid (4000-400 cm^{-1}) and far (<400 cm^{-1}) IR regions (Stuart, 2005). The positions of IR absorption on the spectrum are typically presented as wavenumbers (cm^{-1}) or wavelengths (μm), with wavenumber defined as the number of waves per unit length (i.e. cm) (wavenumber = 1/wavelength) (Settle, 1997; Stuart, 2005). Information produced from the measurement of IR absorption is often presented as a spectrum with wavenumber on the x-axis and absorption intensity on the y-axis (Settle, 1997).

The mid-IR is useful for structural and quantitative determination since IR-active functional groups have characteristic absorbance within this region. The mid-IR spectra

between 4000 and 400 cm^{-1} can be segmented into four broad regions: the X-H stretching region (4000-2500 cm^{-1}), the triple bond region (2500-2000 cm^{-1}), the double bond region (2000-1500 cm^{-1}), and the fingerprint region (1500-400 cm^{-1}). The fingerprint region is considered useful for identification rather than relying upon individual band assignment (Günzler and Gremlich, 2002).

The general principle underpinning biomedical IR spectroscopy is that all molecules within a sample are in continuous vibration at temperatures above absolute zero (Settle, 1997; Maziak et al., 2007). When a sample is exposed to IR radiation, the IR active molecules within that sample absorb light when the IR radiation energy matches the energy of specific molecular vibration (Maziak et al., 2007). The IR spectrometer measures the amount of radiation absorbed at a specific wavenumber and displayed as absorption bands on an IR spectrum. The IR spectrum of small molecules will be simple and consist of well-defined absorption bands that reflect their chemical structure. In contrast, complex samples (such as the biological samples) will generate IR spectrum consist of numerous overlapping absorption bands at different wavenumbers (Vijarnsorn et al., 2006; Maziak et al., 2007).

1.5.1.2 Sampling presentation

There are a number of various IR spectroscopic sampling techniques. Two of the most common are transmission-IR and attenuated total reflectance (ATR) spectroscopy.

1.5.1.2.1 Transmission infrared spectroscopy

In the transmission sampling technique, the sample is directly placed into the path of IR beam, the transmitted energy is measured, and a spectrum is generated (Sun, 2009; Smith, 2011). Although transmission IR spectroscopy has been used for decades in different fields including chemistry, medicine, biology, and geology (Stuart, 2005), technical difficulties are commonly encountered that have made a routine collection of high-quality spectra in the field situation difficult (Oberg and Fink, 1998). These difficulties include practical issues associated with filling and cleaning short-pathlength cells (for spectroscopy of liquid samples), uncertainties in optical pathlength (for spectroscopy of dried films), and the time required for sample submission and preparation (Sun, 2009; Smith, 2011).

1.5.1.2.2 Attenuated total reflectance infrared spectroscopy

ATR has emerged as one of the most commonly used IR spectroscopic sampling techniques, with applications addressing various clinical diagnostic problems in human and veterinary medicine (Ellis et al., 2002; Baldauf et al., 2006; Gupta et al., 2006). The ATR sampling technique measures the changes that occur to the totally internally reflected IR beam after it comes into contact with a sample (Sun, 2009; Smith, 2011). ATR spectroscopy is a versatile and powerful technique for easy infrared sampling. In contrast to transmission IR spectroscopy, ATR has the advantage of reduced sample preparation time because the sample is simply diluted and applied directly to the sensor. ATR is relatively impervious to variations in sample thickness, making a collection of high-quality spectra easier (Fahrenfort, 1961). Therefore, ATR technique is useful for

sampling the surface of smooth materials that either too thick to too opaque for transmission IR spectroscopy. To ensure the collection of high-quality and representative spectra, the sample surface must be highly homogeneous and in close contact with ATR crystal surface (Karoui et al., 2010). Most recently, robust, small footprint ATR equipment has been manufactured that is ideally suited for field use on the farm, veterinary clinic or small laboratory (Seigneur et al., 2015).

1.5.1.3 Spectral data analysis

IR spectrum band positions, intensities, and shape, represent the chemical constituents of the tested sample. Whereas wavenumber band positions give information about the molecular structure of chemical compounds in the sample, the intensities of the bands are related to the concentrations of these compounds (Karoui et al., 2010). The challenge is to extract those portions of the information that will provide the clinically most useful data. The most successful approach to extracting qualitative, quantitative or structural information from IR spectra is to use chemometric methods. The commonly used procedures for the analysis of IR spectra can be divided into three main groups of techniques as follows:

1- **Spectral pre-processing**

Pre-processing of IR spectral data is often important to obtain reasonable results whether the analysis is concerned with classification or building a good and robust quantitative analytical method. The main goal of spectral pre-processing is to amplify useful information and to eliminate errors or noise introduced by nature, treatment and handling of the sample. The pre-processing techniques can

be divided into two main categories: **1-** Reference-independent techniques, which include scatter correction methods (multiplicative signal correction (MSC), standard normal variate (SNV), normalization and baseline correction) and derivation methods (finite difference, Savitzky-Golay, and Norris-Williams) (Savitzky and Golay, 1964; Barnes et al., 1989). **2-** Reference-dependent techniques, which include orthogonalization, optimized scaling, and net analyte pre-processing. In this research project techniques used included smoothing, Savitzky-Golay derivation, normalization, and baseline correction (Karstang and Manne, 1992; Goicoechea and Olivieri, 2001; Westerhuis et al., 2001).

2- Qualitative (classification) methods

Qualitative analysis implies the classification of samples according to their IR spectral patterns. The IR spectral classification methods can be divided into two categories: **1-** Unsupervised classification approaches (samples are classified without a prior knowledge, except the spectra) either with or without spectral preprocessing (e.g. principal component analysis (PCA)). PCA is commonly used to reduce spectral data sets into a small number of new orthogonal variables, from which a score for each sample is calculated. Graphical display of these scores often reveals patterns or clustering within the data set because similar samples are expected to locate close to each other (Martens and Naes, 1992; Martens and Martens, 2001).

2- Supervised classification (samples are classified with prior knowledge of their class membership). A classification model is developed on a training subset of

samples (ideally randomly selected) with known categories then the model performance is evaluated by comparing the classification predictions to the true class membership of the subset of samples reserved for independent validation (Massart, 1988). This category includes several methods such as canonical correlation analysis (CCA), linear discriminant analysis (LDA), hierarchical cluster analysis (HCA), factorial discriminant analysis (FDA), artificial neural networks (ANN), and discriminant partial least squares (PLS-DA).

3- Quantitative (regression) methods

The use of IR spectra for quantification purposes may be achieved by regression techniques such as principal component regression (PCR) and partial least squares (PLS) regression (Adams, 2004). These methods are used to explore and establish relationships between IR spectra generated for each sample of interest and their corresponding reference concentration values (Geladi and Kowalski, 1986). Selection of the particular quantitative method depends on not only the prediction ability but also the cost of calibration in terms of sample objects and reference data needed to build and optimize the calibration models (Perez-Guaita et al., 2013).

PLS regression is the most common method employed for regression and modeling of IR spectral data (Wold et al., 2001). For reliable estimation of the predictive ability of PLS model, the sample data set, including the spectra matrix (**X**) and the reference concentration (**Y**), are generally divided into a randomly selected calibration and validation data subsets (Geladi and Kowalski, 1986). The

calibration data set is used to build professional PLS models and for selection of the best parameters and condition. For this purpose, a cross-validation based approach is used, and the root mean square error of cross validation (RMSECV) calculated (Riley et al., 2007). After selection of the best preprocessing parameters, the model is validated using an independent set of data obtained from samples included in the validation set, which were not employed in building and optimizing the model, so as to obtain a realistic value of the prediction error.

1.5.1.4 Advantages of infrared spectroscopy

Infrared spectroscopy has numerous advantages for the quantitative and qualitative characterizations of biological samples. Versatility is one of the main strengths of IR spectroscopy as almost all forms of the biological samples including gas, liquid or solids are active in the IR range and can therefore, be quantifiable by IR methods (Vijarnsorn et al., 2007). An additional advantage of IR methods is that the analysis can be performed directly without the need for any reagents, and sample preprocessing and the results can be available within minutes from a serum or plasma sample (Shaw et al., 2000). These advantages make IR methods well suited for point-of-care use in diagnostic laboratories, emergency departments and veterinary clinics (Low-Ying et al., 2002; Riley et al., 2007). Cost-effectiveness is another advantage of IR methods compared with other analytical methods including low per-sample cost, inexpensive repeat testing, and the measurement of different parameters can be determined from single testing (Riley et al., 2007). Moreover, only a small sample volume is needed to perform IR analysis. Sustainable features of IR spectroscopy are

important in saving reagents and reducing waste generation. The recent development of compact small, portable, automated instrumentation opens the door to use IR spectroscopy under the field settings (Brandstetter et al., 2013). The aforementioned advantages underpin the application of IR spectroscopy as a promising analytic technique in the human and veterinary health care without the creation of economic and environmental problems.

1.5.1.5 Limitations of infrared spectroscopy

Limited selectivity is probably the most important drawback of the IR spectroscopic quantification of clinical parameters in biological samples (Perez-Guaita et al., 2013). All the components and organic analytes in a sample represents absorption in the IR region, which could interfere with the target analyte. Because of that, multivariate data analysis is normally required to extract the desired analytical information from the sample spectra (Perez-Guaita et al., 2014). Recently, some efforts have focused on separation of some interferents (sample preparation) before sample measurement, but this may lead to the loss of some of the principle benefits of the IR-based analytic methods mentioned before (Perez-Guaita et al., 2014).

1.5.1.6 Practical application of infrared spectroscopy

Infrared spectroscopy has become a promising technique for the quantitative and qualitative analysis of clinical samples in both human and veterinary medicine.

1.5.1.6.1 IR spectroscopy in human medicine

IR spectroscopy is already a well-established technique for quantification of different parameters in a variety of human clinical samples; including, liquid, such as whole blood, serum, plasma, urine, breast milk and synovial fluid; solids, such as skin, hair, urinary stones and tissues (Perez-Guaita et al., 2014). In combination with multivariate data analysis the technique has successfully been use to quantify various biochemical parameters in blood, serum and plasma such as glucose, total proteins, albumin, cholesterol, fibrinogen, triglyceride, lipoproteins and immunoglobulins (Janatsch et al., 1989; Petibois et al., 2001; Rohleder et al., 2005). In urine samples, IR-based methods have been used for quantification of urea, creatinine, uric acid, phosphate and sulfate (Heise and Marbach, 1998; Hoşafçı et al., 2007). IR methods have also been used for the characterization of urinary calculi (Basiri et al., 2012). Recently, IR spectroscopy has been investigated for its use in the diagnosis of arthritis via synovial fluid analysis (Eysel et al., 1997; Jackson et al., 1997) and for analysis of excised tissues or isolated cells (Jackson et al., 1997). Several studies indicating the potential of IR spectroscopy to detect changes in biochemical composition of cells at a molecular level including those associated with cervical, esophageal, and gastric neoplasia, and certain leukemias (Li et al., 2005; Maziak et al., 2007).

1.5.1.6.2 IR spectroscopy in veterinary medicine

The applications of IR spectroscopy developed for human medicine can be utilized to solve problems in veterinary medicine. The advantages above of the IR-

spectroscopy open the door for possible applications in veterinary medicine in both laboratory and field settings.

In veterinary medicine, analytical methods based upon IR spectroscopy have been proven suitable as reagent free tools for measurement of fat, protein, lactose and urea in milk (Rutten et al., 2011a; Rutten et al., 2011b), determination of feed composition and the kinetics of nutrient degradation in the rumen (Herrero et al., 1997; Ohlsson et al., 2007), and screening for metabolic diseases such as ketosis (Hansen, 1999). Other studies have investigated the potential of IR spectroscopy as a diagnostic tool for the measurement of somatic cell count (SCC) in milk (Tsenkova et al., 2001) and for the detection of infectious diseases such as foodborne pathogen (Davis and Mauer, 2010), Johne's disease and bovine spongiform encephalopathy (BSE) (Martin et al., 2004; Menze et al., 2007). Applications of IR spectroscopy in veterinary medicine have been extended to detection of musculoskeletal disorders such as osteoarthritis (Kidd et al., 2001) and osteochondrosis (Vijarnsorn et al., 2006; Vijarnsorn et al., 2007). The technique has also been used for the identification of urinary calculi in canine, feline and equine species (Diaz-Espineira et al., 1997; Escolar and Bellanato, 2003).

1.5.2 ZAPvet bovine IgG test

The ZAPvet Bovine IgG test is a fast and technically simple on-farm test to confirm the adequate transfer of passive immunity (APTI) in calves. The ZAPvet Bovine IgG Test uses two visual lines, a test line, and a reference line, to provide a semiquantitative determination of the concentration of bovine IgG using whole blood, plasma, or serum samples. The ZAPvet Bovine IgG Test was developed using calibrators

with turbidimetric-determined concentrations. The ZAPvet test is inexpensive and rapid; it requires no instruments, reagents, or buffers, and therefore, can be performed anywhere and at any time.

1.6 Summary

Measurement of bovine serum and colostral IgG concentrations is important for detection of FTPI and colostrum quality. Several tests exist for the measurement of bovine serum and colostral IgG concentrations. Radial immunodiffusion assay (RID) is the reference test for the quantitative measurement of IgG. While RID generates quantitative IgG data, it has significant drawbacks. Other tests have been used to determine IgG levels, but these are primarily best used as screening tests for the diagnosis of FTPI, with very good diagnostic sensitivity but often only moderate diagnostic specificity when compared to RID. Therefore, in effort to accurately detect of FTPI in calves and evaluate colostrum quality from their dams, there is still a need to provide veterinary clinics and producers with a rapid, cost-effective test with a very good to excellent sensitivity and specificity for quantification of IgG.

1.7 Thesis objectives

The overall objectives of this thesis were (1) to develop and validate an IR-based assay for rapid quantification of bovine serum and colostrum IgG concentrations, and (2) to validate the novel and current on-farm tools for assessing FTPI in dairy calves and colostrum quality of dairy cows.

Specific objectives were:

- Chapter 2: to develop a quantitative assay for bovine serum IgG, based upon the use of transmission-IR spectroscopy in combination with a partial least squares regression method (PLS) and to investigate its potential use for diagnosis of FTPI in dairy calves.
- Chapter 3: to investigate the potential use of ATR spectroscopy, in combination with multivariate data analysis, for the rapid quantification of IgG concentrations in bovine serum, and for the diagnosis of FTPI in dairy calves.
- Chapter 4: to investigate the utility of previously built partial least squares (PLS) models for quantifying IgG for a new set of samples and to compare the performance of transmission-IR and ATR-IR spectroscopic approaches as the basis to predict IgG levels in bovine serum, and to diagnose FTPI in dairy calves.
- Chapter 5: to develop a transmission IR-based analytical method for the quantification of bovine colostral IgG, and to demonstrate the utility of the new method for the assessment of poor quality colostrum
- Chapter 6: to describe the test characteristics of the initial version of a ZAPvet Bovine IgG test against RID, and measure inter-observer variations for assessing FTPI in dairy calves.
- Chapter 7: to determine the sensitivity, specificity and accuracy of both digital and optical refractometers for assessing the success of transfer of passive immunity in dairy calves, and to evaluate the agreement between two types of refractometers.

- Chapter 8: to determine the sensitivity, specificity, and accuracy of both digital and optical Brix refractometers for assessing colostrum quality in dairy cows, and to evaluate the agreement between the two refractometers.

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CHAPTER 2

MEASUREMENT OF SERUM IMMUNOGLOBULIN G IN DAIRY CATTLE USING FOURIER-TRANSFORM INFRARED SPECTROSCOPY: A REAGENT FREE APPROACH

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2.1 ABSTRACT

Simple, rapid and cost-effective methods are sought for measuring immunoglobulin G (IgG) concentrations in bovine serum, which can be applied for diagnosis of failure of transfer of passive immunity (FTPI). The aim of the present study was to investigate the potential use of Fourier-transform infrared (FTIR) spectroscopy, with partial least squares (PLS) regression, to measure IgG concentrations in bovine serum. Serum samples collected from calves and adult cows were tested in parallel by radial immunodiffusion (RID) assay and FTIR spectroscopy. The sample IgG concentrations obtained by the RID method were linked to pre-processed spectra and divided into two sets: a combined set and a test set. The combined set was used for building a calibration model, while the test set was used to assess the predictive ability of the calibration model, resulting in a root mean squared error of prediction (RMSEP = 307.5 mg/dL).

The concordance correlation between the IgG measured by RID and predicted by FTIR were 0.96 and 0.93 for the combined and test data sets, respectively. Analysis of the data using the Bland-Altman method did not show any evidence of a systematic difference (bias) between FTIR and RID methods for measurement of IgG. The clinical applicability of FTIR for diagnosis of FTPI was evaluated using the entire data set and had a sensitivity of 0.91 and specificity of 0.96 using RID as the reference standard. The FTIR method described herein demonstrates potential as a rapid and reagent-free tool for quantification of IgG in bovine serum, for use in diagnosis of FTPI in calves.

2.2 INTRODUCTION

The lack of trans-placental transfer of immunoglobulin (Ig) in ruminant species means that dairy and beef calves are born without significant circulating IgG. They are, therefore, dependent upon ingestion and absorption of maternally derived antibodies from colostrum (Baintner, 2007) during the 12–24 h period after parturition for passive immunity to infection (Butler, 1983; Godden, 2008). Failure of transfer of passive immunity (FTPI) is a condition that occurs as a result of inadequate ingestion or absorption of IgG (< 1,000 mg/dL) and is considered a major predisposing risk factor for neonatal morbidity and mortality, associated with gastroenteritis, pneumonia and septicemia (Tyler et al., 1996b; Godden, 2008). The effects of FTPI might extend beyond the neonatal period in heifer calves, affecting long-term productivity and resulting in decreased milk yield and increased culling rates during first lactation (DeNise et al., 1989; Heinrichs and Heinrichs, 2011). The reported prevalence of FTPI in North America is relatively high, estimated at 19% to 40% in dairy calves (Nocek et al., 1984; Dairy, 2007) and has major economic consequences for dairy producers (Trotz-Williams et al., 2008). Monitoring of calves for FTPI can identify herd management deficiencies, and ensure timely detection and implementation of interventional measures, which can reduce the risk of infectious diseases (Furman-Fratczak et al., 2011).

Several diagnostic tests have been developed to measure circulating IgG in neonatal calves (Tyler et al., 1996b; Parish et al., 1997; Weaver et al., 2000). Direct methods include enzyme-linked immunosorbent assays (ELISA), the radial immunodiffusion (RID) assay (Filteau et al., 2003) and an automated turbidimeteric

immunoassay (Alley et al., 2012), while indirect methods include the sodium sulfate turbidity test, zinc sulfate (ZnSO4) turbidity test, glutaraldehyde coagulation test and refractometry. These screening tests have been widely used as an aid for diagnosis of FTPI in calves. Key limitations of the indirect methods and ELISA are that they suffer from moderate to poor sensitivity and specificity compared to RID; moreover indirect methods are qualitative, rather than quantitative (Calloway et al., 2002; McGuirk and Collins, 2004; Wallace et al., 2006). RID is considered to be the 'reference standard' test for quantification of IgG in bovine serum (McBeath et al., 1971). While accuracy is generally good, the RID method has some drawbacks in that it takes 18–24 h to obtain results, requires technical skill to perform, is not amenable to automation (Liu et al., 2007), utilizes reagents with a limited shelf life, and is often more expensive than the indirect assay methods (Riley et al., 2007; Bielmann et al., 2010). Therefore, it would be desirable to develop a sensitive and specific test for bovine IgG that is simple, rapid, practical and cost-effective.

Fourier-transform infrared (FTIR) spectroscopy has emerged as a useful tool in modern analytical chemistry. In FTIR transmission spectroscopy, a beam of electromagnetic radiation encompassing the mid-infrared (IR) spectral range (2.5 - 25 microns, or 4000 - 400 cm^{-1}) is passed through a sample of interest (e.g. serum), where each of the constituent molecular species absorbs IR radiation at a specific set of wavelengths, which can be measured (Riley et al., 2007). With no requirement for physical separation of the components, and no requirement for additional reagents, such tests are simple, quick, and inexpensive (Shaw et al., 1998; Shaw and Mantsch, 1999; Shaw and Mantsch, 2000). Small portable FTIR spectroscopy units have been developed,

which would allow this technique to be undertaken on farms or in veterinary clinics in the future.

Previous clinical studies have demonstrated that serum total protein, glucose, albumin, triglyceride, cholesterol, and urea can be quantified from a single FTIR spectroscopic measurement (Ward et al., 1989; Budinova et al., 1997; Shaw et al., 1998). More recently, the same approach has been used to quantify serum or plasma IgG in veterinary species including the horse and alpaca (Riley et al., 2007; Burns et al., 2014; Hou et al., 2014). In the dairy industry, FTIR spectroscopy has been widely used for nutrient analysis of milk (Rutten et al., 2011a; Rutten et al., 2011b) and screening of dairy cows for metabolic diseases such as ketosis (Hansen, 1999). However, to date, there are no reports of this technique being used to quantify IgG in bovine serum. The aims of the present study were to develop a quantitative assay for bovine serum IgG, based upon use of FTIR spectroscopy in combination with a partial least squares (PLS) regression method and to investigate its potential use for diagnosis of FTPI in dairy calves.

2.3 MATERIALS AND METHODS

2.3.1 Samples

A total of 250 serum samples from Holstein-Friesian calves (n = 208, eight of which were prior to colostrum ingestion) and adult dairy cows (n = 42) were used in the study. Samples had been collected for companion studies (Fecteau et al., 1997a; Fecteau et al., 1997b) and were stored frozen at -80 °C at the University of Prince Edward Island

before use. All samples were tested in parallel using a radial immunodiffusion assay (RID) and by FTIR spectroscopy.

2.3.2 Radial immunodiffusion assay

Serum samples were thawed at room temperature (20–24 °C) and vortexed for 10 s. Subsequently, IgG was measured using the Bovine IgG RID Kit (Triple J Farms; working range of 196 to 2.748 mg/dL), except for eight samples that were collected prior to colostrum ingestion, which were measured using the Bovine Ultra Low Level IgG RID Kit (Triple J Farms; working range of 10 to 100 mg/dL). The RID assays were performed according to manufacturer's recommendations, using 5 μL of undiluted serum in each well, with the manufacturer's standard samples used on all RID assays.

The diameters of precipitated rings were measured after 18–24 h of incubation at room temperature, using a handheld caliper. Each of the serum samples and assay standards was tested in replicates of five and mean values calculated. The assay standards were used to construct a calibration curve that was used to determine IgG concentrations for the test samples. Serum samples with IgG concentrations outside the manufacturer stated performance range for the assay (above) were excluded from further analysis (n = 50; 28 calves; 22 cows).

2.3.3 Fourier-transform infrared (FTIR) spectroscopy

Thawed serum samples were diluted (1:1) with deionized sterile water and vortexed at a maximum of 2700 rpm for 10 s to homogenize the samples. After dilution, 10 μL aliquots were spread evenly onto 5 mm diameter wells within an adhesive-masked,

96-well silicon microplate (Riley et al., 2007). For each sample, six replicate spectra were recorded to assess precision. The loaded microplates were allowed to dry at room temperature (20–24 °C) for 2 hours to evaporate water, resulting in thin dried films. For collection of FTIR spectra, the microplates were inserted into a multisampler (HTS-XT Autosampler, Bruker Optics) interfaced with a Bruker FTIR spectrometer (Tensor 37, Bruker Optics) equipped with a deuterium tryglycine sulfate detector and controlled by proprietary software (OPUS version 6.5, Bruker Optics).

Absorbance spectra were collected at wavenumbers between 4000–400 cm^{-1} with a nominal resolution of 4 cm^{-1} with 512 scans collected for data acquisition. An empty well served as the background reference for each microplate. A total of 1.500 (250 × 6) spectra were collected and converted into a printable format (PRN) (GRAMS/AI version 7.02, Thermo Fisher Scientific). The PRN format spectral data were imported into MATLAB version R2012b (MathWorks) and further data analysis was performed using scripts written by the authors.

2.3.4 Spectra pre-processing

The infrared spectra were pre-processed using the Savitzky-Golay method (2nd order polynomial function with nine points) to generate first order derivative spectra (Savitzky and Golay, 1964). The derivative spectra then were normalized using a vector normalization procedure, in which the square root of the sum of squared intensities of each spectrum over the wavenumber range of 1600–1800 cm^{-1} were calculated, and then the intensity at each wavenumber across the full measured spectral range was divided by this factor. Spectra were then cut and truncated to include only the 3700–2600 cm^{-1} and

1800–1300 cm^{-1} wavenumber regions, which exhibited the strongest absorptions in the original spectra. Subsequently, spectrum outlier detection was performed using Dixons Q-test (Dean and Dixon, 1951; Rorabacher, 1991) at each wavenumber. Spectra were designated as outliers if more than 50% of absorbance values were outside the 95% confidence level; these outliers were excluded from further analysis. The average spectrum of each sample (after removal of outlier spectra if applicable) was used for subsequent analysis.

2.3.5 Calibration model building

The sample IgG concentrations obtained by the RID method that were within the reference range (n = 200) were linked to their corresponding pre-processed infrared spectra and the samples divided into two sets. The test set (n = 67) were identified by ordering all of the spectra according to their corresponding RID IgG levels (minimum to maximum) and selecting every third spectrum. Thus, the test set encompassed the full range of IgG values as determined by RID. The remaining 'combined' set (n = 133) was used for model development, once randomly split into a training (n = 67) and a validation data set (n = 66).

PLS regression was applied to the training set to develop trial calibration models with up to 30 PLS factors, and the analytical accuracy of each trial model was estimated as the sum of squares of the errors for the validation set. This procedure was repeated 10,000 times (including new randomly assigned splits of the combined data set into new training and validation sets). The root mean squared error for the Monte Carlo cross-

validation value (RMMCCV) (Richard R. Picard and R. Dennis Cook, 1984; Xu and Liang, 2001) was calculated by the following equation:

$$\mathrm{RMMCCV} = \sqrt{\frac{1}{Nn_{\mathrm{v}}}\sum_{i=1}^{N}\left\|\mathbf{y}_i - \hat{\mathbf{y}}_i\right\|^2}$$

where N denotes the number of repeated procedures (N = 10,000), n_{v} is the number of samples in the validation set (n_{v} = 66), and $\mathbf{y}_i$ / $\hat{\mathbf{y}}_i$ represent the IgG concentrations for the validation set samples as obtained from RID experiments and predicted from the IR spectroscopic data, respectively. The notation $\|\bullet\|$ denotes the operator of Euclidean norm. The optimal number of PLS factors was chosen as the one giving the lowest RMMCCV value. Once the number of PLS factors had been determined, the training set and validation set were combined to build the calibration model.

2.3.6 Performance of the FTIR assay

The test set was used to evaluate the predictive performance of the optimized model. Scatter plot, Pearson correlation coefficient, and concordance correlation coefficient (Lin, 1989) were used. The three criteria were also applied to assess the combined set to examine model fitting. A Bland-Altman plot was used to examine the difference between RID and FTIR values for the test set and thereby assess the interchangeability of the two assays (Altman and Bland, 1983; Martin Bland and Altman, 1986; Bland and Altman, 1995). A paired Student's *t*-test was performed to evaluate

differences between the RID and FTIR methods, with a significant difference recognized at $P < 0.05$. The model's potential utility was further examined using the ratio of predictive deviation (RPD: ratio of the standard deviation of the IgG concentration measured by RID to RMSEP) and the range error ratio (RER: ratio of the range of the IgG concentration measured by RID to RMSEP).

The precision of the new analytical method was also assessed using the test set data. The relative standard deviation (coefficient of variation) was calculated for the IgG concentrations predicted from replicate spectra for each sample, and used as an indicator of precision. For comparison, the relative standard deviation of each sample tested by RID assay was also calculated. These calculations were performed with Stata version 13.0 statistical software (StataCorp).

To evaluate the clinical applicability of FTIR spectroscopy for diagnosis of FTPI, the sensitivity (Se), specificity (Sp) and accuracy were evaluated at a RID cut-off of 1,000 mg/dL (Godden, 2008; Fecteau et al., 2013). The FTPI prevalence (P) for test and entire data were calculated using the same criteria.

2.4 RESULTS

2.4.1 Serum IgG determined by RID assay

The mean and SD for IgG concentrations of the serum samples (n = 250) as obtained by RID were 1,826 ± 1,787 mg/dL (range 5.5 to 10,945 mg/dL). Serum samples with an IgG concentration greater than the highest standard (3,000 mg/dL) were excluded from further analysis because they were considered outside the biologically relevant

range for the target condition, and exceeded the range over which the manufacturers had validated the reference method. The mean and SD for IgG concentrations of the remaining samples (n = 200) as measured by RID were 1,122 ± 866 mg/dL (range 5.5 to 2,983 mg/dL).

2.4.2 FTIR spectra

The infrared spectra of serum showed absorption peaks characteristic of biological samples with a broad, strong absorption band centered at 3300 cm^{-1}, attributed to N-H stretching vibration (amide A), and other strong absorption bands around 1650 and 1550 cm^{-1}, resulting from C=O stretching vibrations (amide I) and N-H bending vibrations (amide II), respectively (Shaw et al., 1998; Shaw and Mantsch, 2000) (Figure 2.1).

2.4.3 Calibration model

The optimal number of PLS factors for FTIR-IgG concentration measurement was determined to be 15, based on the lowest RMMCCV (372 mg/dL). This model was obtained using data from the first derivative spectra with nine points smoothing (Figure 2.2); vector normalized to the 1600–1800 cm^{-1} region. The performance characteristics 15-factor model, as applied to both the test set and combined calibration/validation set, are presented in Table 2.1.

The level of agreement between IgG concentrations measured by RID and those predicted by FTIR spectroscopy for the combined and test sets are represented by scatter plots (Figure 2.3). The plots show similar dispersions, with no significant under-fitting and over-fitting problems, indicating the calibration model is well-determined. The

Pearson correlation coefficients (r) for the combined and test sets were 0.97 and 0.94, respectively. The concordance correlation for the combined set was 0.96 and test set was 0.93 (Figure 2.4). The plots show that at high IgG concentrations, the level of agreement between RID and FTIR spectroscopy is less than that at low concentrations. Agreement between RID and FTIR spectroscopy was further assessed using the Bland-Altman plot (Figure 2.5). The average of the differences between concentrations obtained by RID and FTIR was 0.15 mg/dL, indicating no obvious systematic errors (bias) between the two methods. The 95% confidence interval range was -607 to 607 mg/dL; a spread that is small enough to suggest that the FTIR assay for serum IgG has accuracy that is comparable to the RID method. The paired Student's t-test showed no significant difference ($P > 0.05$) between the mean of IgG concentrations measured by RID and FTIR assay, in agreement with the Bland-Altman method. Finally, the RPD and RER were estimated at 2.9 and 9.7, respectively.

2.4.4 Precisions of RID and FTIR spectroscopy methods

Figure 2.6 shows the plots of relative standard deviations for RID and FTIR spectroscopy methods. No obvious correlation between IgG concentrations measured by RID and its relative standard deviations (Figure 2.6a) was observed. However for the FTIR spectroscopy method, the relative standard deviation was large when IgG concentration was low but decreased with increasing IgG concentration (Figure 2.6b). Overall, the RID assay had better precision than FTIR assay in this study.

2.4.5 Evaluation of the clinical utility of the FTIR assay

The number of samples with IgG concentrations below a threshold value of 1,000 mg/dL for FTPI, based on the RID assay, for the test and entire data sets were 34 and 102, respectively, giving a true prevalence for FPTI of 51%. Table 2.2 presents the Se, Sp and accuracy of the FTIR-based analytical method for the test set and separately for the entire data set. Within the entire data set, there were four false positive (FP) and nine false negatives (FN) classifications identified. The 'true' IgG concentrations (measured by RID) were close to 1,000 mg/dL for most FN samples, indicating only partial FTPI. Others have defined a value as low as 800 mg/dL as constituting partial FTPI (Perino et al., 1993; Fecteau et al., 2013). The IgG concentrations predicted by the FTIR assay for FP samples were also close to the diagnostic threshold of 1,000 mg/dL (Table 2.3).

2.5 DISCUSSION

The IgG concentrations in serum samples recruited for the encompassed the full range of values for which the reference method (RID) has been validated. While the primary target population for application of the FTIR technology is neonates, to achieve a wide range of values for analysis, adults were also included. This ensured that the calibration model would be effective for the measurement of IgG concentrations across the range of clinically relevant values (Murray, 1986).

The performance of the FTIR-based IgG analytical method was evaluated using a number of methods for critical evaluation, rather than a single approach. All metrics confirmed good agreement with an independent test set (the reference RID analyses). A

recent study (Zhang et al., 2013) employing near-infrared spectroscopy for prediction of human IgG concentrations reported a correlation coefficient of 0.95, which is close to the value in the present study. Use of FTIR spectroscopy for measurement of bovine IgG was more accurate (superior overall correlation and concordance) than similar methods for measuring serum IgG in horse and alpaca (Riley et al., 2007; Burns et al., 2014; Hou et al., 2014).

The relatively poor agreement between the FTIR spectroscopy results and IgG concentrations determined by RID assay at high IgG concentrations parallels similar findings for IgG serum/plasma assays developed for other species (Burns et al., 2014; Hou et al., 2014). However, this is observation not particularly relevant for diagnosis of FTPI in farm animal practice, since it occurred at concentrations well above the FTPI threshold value of 1,000 mg/dL (Godden, 2008; Fecteau et al., 2013). The final calibration model was built without the inclusion of samples with IgG concentrations above the highest standard used in the RID. It is unlikely that such samples would commonly occur in calves being screened for FTPI, and almost certainly the case that the IR-based analytical method would properly categorize them as having IgG levels well above the diagnostic cut-off.

Williams and Sobering (1996) have reported that an RPD value >2 indicates model prediction that is acceptable for quantification, and values >3 indicative of highly accurate quantitative analysis (Williams and Sobering, 1996). The RPD value for the FTIR-based assay reported here was 2.9, indicative of the latter. In comparison, the RER is a measure of the relationship between the range of values and root mean squared error of the PLS model. Models with a RER values <3 have a little practical utility, whereas

values >10 show that the model has a high level of utility (Williams and Sobering, 1996). The RER value in the current study was 9.7, indicating that the new analytical method has good to high utility. Thus, with a standard deviation equal to three times the prediction error and a range of reference data almost 10 times the prediction error, this method can be used to accurately quantify bovine serum IgG levels.

The clinical diagnostic accuracy of the FTIR spectroscopy technique was assessed an IgG concentration <1,000 mg/dL as a threshold value, below which FTPI is diagnosed (Godden, 2008). The FTIR assay showed excellent sensitivity and specificity, when compared with data reported for other bovine FTPI testing methods (Tyler et al., 1996a; Tyler et al., 1996b; Lee et al., 2008). The sensitivity of the FTIR assay (Se = 0.91) was superior to that of glutaraldehyde coagulation test (Se = 0.41), similar to that of ELISA (Se = 0.98), sodium sulfate assay (Se = 0.85) and refractometer (Se = 0.85), and lower than that zinc sulfate assays (Se = 1.00). Specificity of the FTIR assay (Sp = 0.96) was similar to that of ELISA (Sp = 0.91), sodium sulfate assay (Sp = 0.87) and refractometry (Sp = 1.00), and superior to that of zinc sulfate assay (Sp = 0.52) (Tyler et al., 1996a; Tyler et al., 1996b; Lee et al., 2008). The majority of the FTPI cases were diagnosed correctly by FTIR spectroscopy, with a relatively low level of false negative and false positive classification. Moreover, most of the misclassified samples had IgG concentrations measured by RID that were close to 1,000 mg/dL, indicating only partial FTPI, perhaps posing a lower risk of morbidity and mortality (Lee et al., 2008; Fecteau et al., 2013).

2.6 CONCLUSIONS

The present study evaluated the usefulness of FTIR and multivariate data analysis as the basis for an IR-based analytical method to determine IgG concentrations in bovine serum samples and assess FPTI in calves. FTIR spectroscopy in combination with PLS regression is a feasible approach for the rapid quantification of IgG concentrations in bovine serum, with good to excellent accuracy, specificity, and sensitivity. A prospective study to assess failure of passive immunity transfer in calves is warranted.

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Table 2.1. Metrics characterizing the final PLS model for the quantitative IR spectroscopy-based determination of IgG concentrations in bovine serum samples.

Data sets	PLS model parameters and performance characteristics [a]								
	n	PLS factors	IgG population			*r*	RMSEC/ RMSEP	RPD	RER
			Mean	SD	Range				
Combined	133	15	1,117	864	2,975	0.97	239.5	-	-
Test	67	15	1,132	876	2,977	0.94	307.5	2.9	9.7

[a] Mean = average of RID data (mg/dL); SD = standard deviation of RID data (mg/dL); range = difference (highest minus lowest) of RID data (mg/dL); *r* = Pearson correlation coefficient; RMSEC = root mean squared error of calibration (mg/dL); RMSEP = root mean squared error of prediction (mg/dL); RPD (ratio of predictive deviation) = SD divided by RMSEP; RER (range error ratio) = range divided by RMSEP.

Table 2.2. Characteristics of FTIR spectroscopy-based IgG assay as a diagnostic test method to determine FTPI [a] in the test (n = 67) and entire data sets (n = 200).

Data sets	Pr	Se (95% CI)	Sp (95% CI)	Accuracy
Test	0.51	0.88 (0.73 – 0.97)	0.94 (0.80 – 0.99)	0.91
Entire	0.51	0.91 (0.84 – 0.96)	0.96 (0.90 – 0.99)	0.94

[a] Calves with serum IgG concentration <1,000 mg/dL (as determined by RID assay) were considered to be affected with FTPI. Pr = prevalence of FTPI; Se = sensitivity; Sp = specificity; all were calculated by comparing FTIR spectroscopy-based assays with their RID-based counterparts.

Table 2.3. Concentration of IgG levels as measured by RID and FTIR spectroscopy-based assays for all FPTI false positive and false negative cases within the entire data set (n = 200).

Data set	False positive		False negative	
	RID (mg/dL)	FTIR (mg/dL)	RID (mg/dL)	FTIR (mg/dL)
Test set	1,201	978	569	1,242
	1,026	923	693	1,091
			886	1,422
			946	1,140
Combined set	1,237	996	811	1,163
	1,132	878	892	1,286
			562	1,046
			967	1,003
			837	1,059

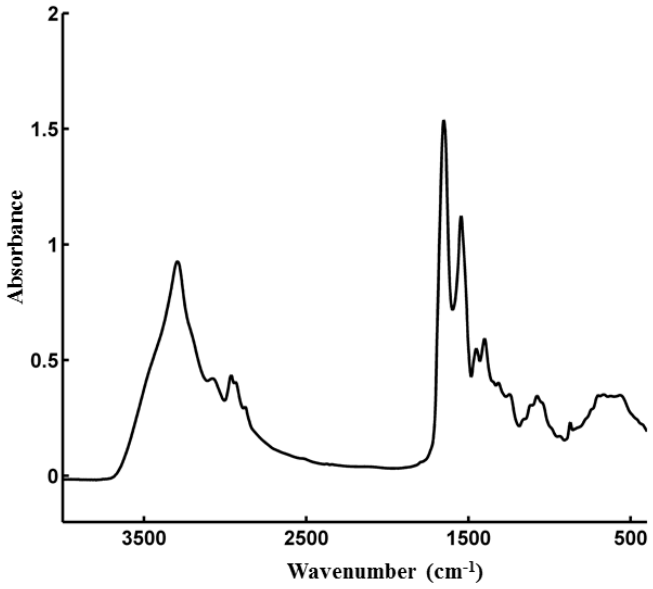

Figure 2.1. Representative infrared spectrum of bovine serum samples over the spectral wavenumber range of 4000 to 400 cm^{-1}. The absorptions centered around 3300, 1650 and 1550 cm^{-1} correspond to protein N-H stretching (amide A), C=O stretching (amide I) and N-H bending (amide II) modes, respectively. (B) First derivatives of FTIR spectra of 250 bovine serum samples (calves and cows).

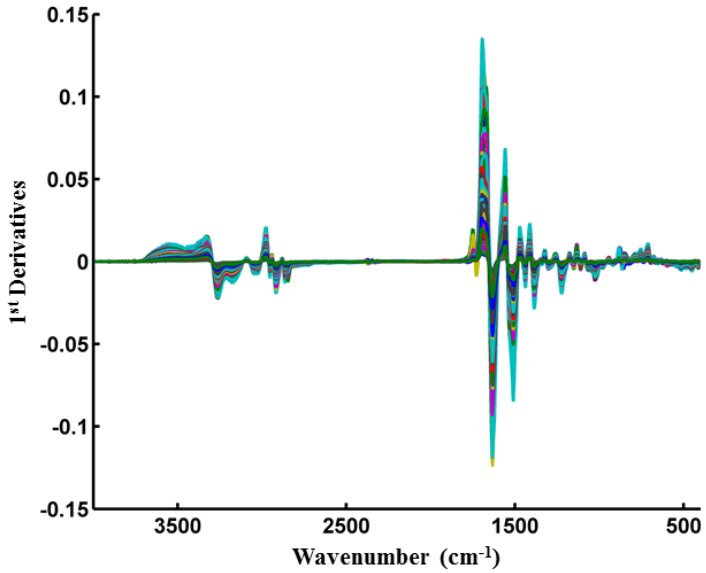

Figure 2.2. First derivatives of FTIR spectra of 250 bovine serum samples (calves and cows).

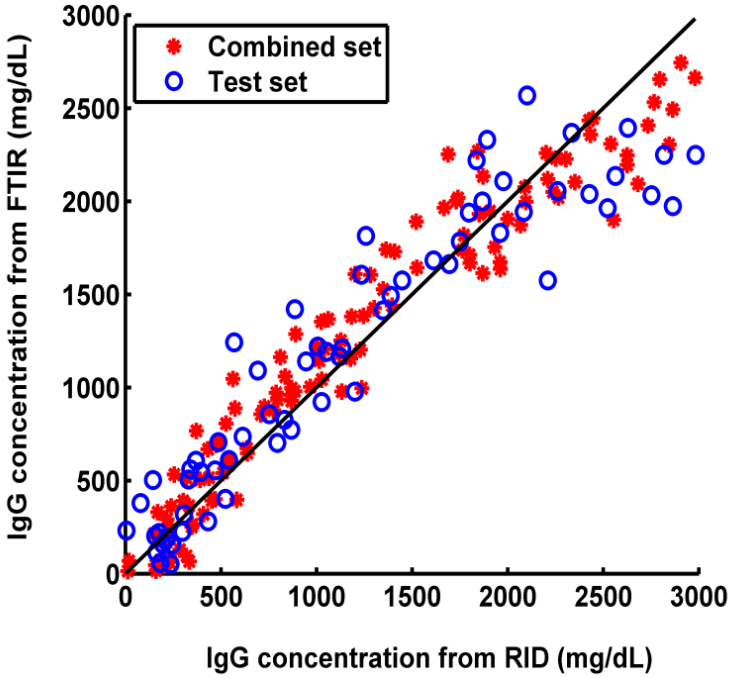

Figure 2.3. Scatter plots comparing IgG concentrations obtained by RID to those obtained using the FTIR spectroscopy-based assay for the combined and test data sets. The two assays were considered comparable based upon the distribution of data points closely around the reference line of 45^{o}.

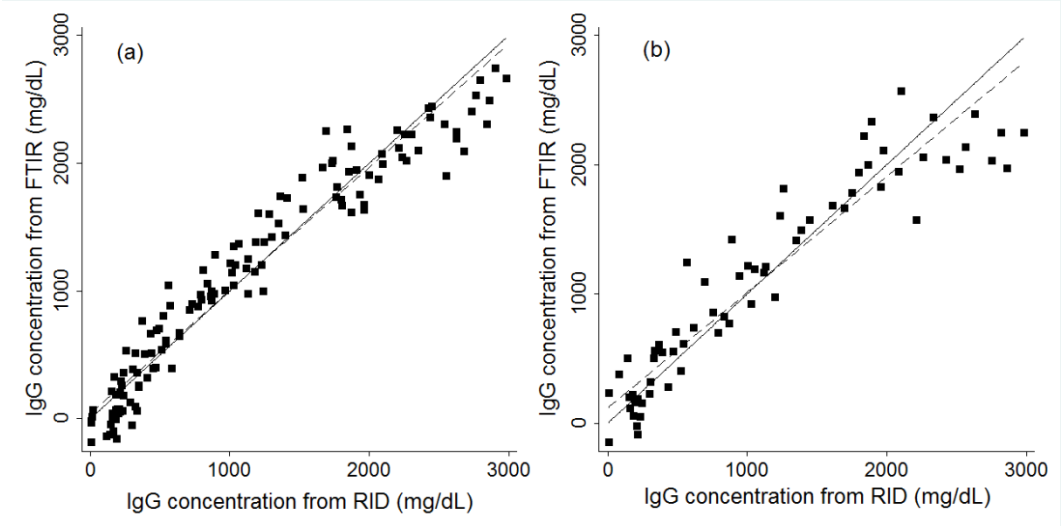

Figure 2.4. Concordance correlation plots comparing IgG concentrations obtained via RID and FTIR spectroscopy-based methods for the (a) combined and (b) test data set. The solid 45^{o} line represents perfect concordance, and the dashed line represents the linear regression line through the observations. The dashed line would overlay the solid line in the case of perfect concordance. Note the level of disagreement between RID and FTIR assays at high IgG concentrations is higher than that at low concentrations.

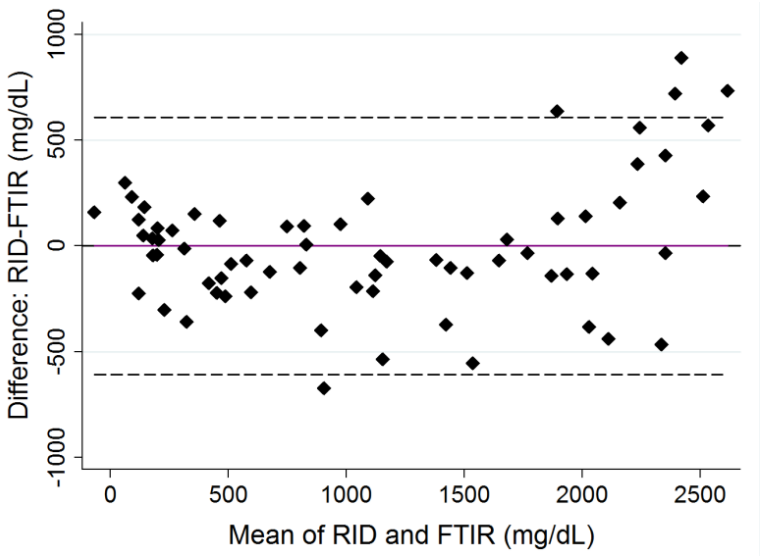

Figure 2.5. Bland-Altman plot of the differences in the immunoglobulin G concentrations measured by RID and FTIR spectroscopy-based methods for the test set (n = 67). The x-axis represents the average of IgG values obtained by RID and FTIR while the y-axis represents the difference (RID minus FTIR). The dashed lines represent the 95% confidence limits of agreement (-607 to 607 mg/dL), and the solid line represents the mean difference between RID and FTIR assay (0.15 mg/dL), indicating no meaningful systematic errors between the two methods.

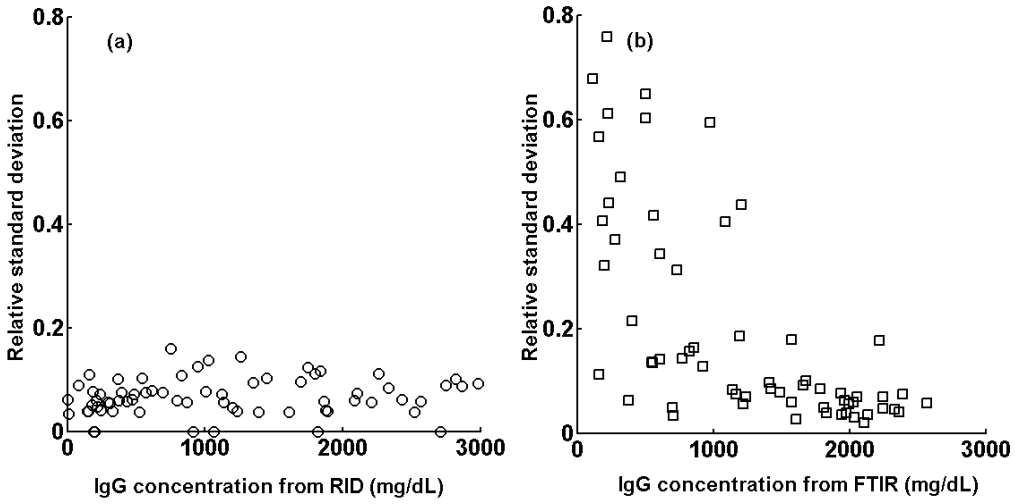

Figure 2.6. Plots of relative standard deviation (coefficient of variation) for the (a) RID and (b) FTIR spectroscopy-based analytical methods. The assay results with the lower relative standard deviation (RID) demonstrated a higher precision than the test method with the higher relative standard deviation (FTIR spectroscopy).

CHAPTER 3

A RAPID FIELD TEST FOR THE MEASUREMENT OF BOVINE SERUM IMMUNOGLOBULIN G USING ATTENUATED TOTAL REFLECTANCE INFRARED SPECTROSCOPY

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3.1 ABSTRACT

Following the recent development of a new approach to quantitative analysis of IgG concentration in bovine serum using transmission infrared spectroscopy, we investigated the potential to measure IgG levels using technology and a device better designed for field use. A method using attenuated total reflectance infrared (ATR-IR) spectroscopy in combination with partial least squares (PLS) regression was developed to measure the bovine serum IgG concentration in the field. ATR-IR spectroscopy has a distinct ease-of-use advantage that would open the door to routine point-of-care testing. Serum samples were collected from calves and adult cows and tested by a reference RID method, and ATR-IR spectra acquired. The spectra were linked to the IgG concentrations from the RID method and then randomly split into two sets: a calibration set and a prediction set. The calibration set was used to build a calibration model, while the prediction set was used to assess the predictive performance and accuracy of the final model. The procedure was repeated for various spectral preprocessing approaches.

For the prediction set, both the Pearson's and concordance correlation coefficients between the IgG measured by RID and predicted by ATR-IR spectroscopy were 0.93. The Bland-Altman plot revealed no obvious systematic bias between the two methods. ATR-IR spectroscopy showed sensitivity for detection of failure of transfer of passive immunity (FTPI) of 88%, specificity of 100% and accuracy of 94% (with IgG concentration <1,000 mg/dL as the FTPI cut-off value). In conclusion, ATR-IR spectroscopy in combination with multivariate data analysis shows potential as an

alternative approach for rapid field quantification of IgG concentrations in bovine serum and diagnosis of FTPI in calves.

3.2 INTRODUCTION

Immunoglobulins are glycoproteins produced by B-lymphocytes, and are a crucial component of the host's adaptive immune system (Rehman et al., 2003). Immunoglobulin G (IgG) is the predominant class of immunoglobulins involved in passive transfer of maternal antibodies to newborn calves via colostrum (Weaver et al., 2000). Failure of transfer of passive immunity (FTPI) occurs when calves fail to ingest and/or absorb sufficient IgG (<1,000 mg/dL) from colostrum (Godden, 2008). FTPI is a major predisposing risk factor for early neonatal losses associated with gastroenteritis, pneumonia, and septicemia (Tyler et al., 1996a; Godden, 2008). Reduced long-term productivity, decreased milk yield, and increased culling rates during the first lactation have also been associated with FTPI (DeNise et al., 1989; Heinrichs and Heinrichs, 2011). The monitoring of IgG levels for timely detection of and response to FTPI are important to reduce productivity losses associated calf-hood diseases (Furman-Fratczak et al., 2011).

A number of methods have been used to measure the IgG concentrations in bovine serum. The most widely accepted reference method used is radial immunodiffusion (RID) assay that provides direct measurement of IgG concentrations in bovine serum (McBeath et al., 1971). However RID has significant drawbacks, including the time it takes to obtain results (18 – 24 h), utilization of labile reagents, high cost

(Riley et al., 2007; Bielmann et al., 2010), and discrepancies among RID kits due to inaccuracies associated with the internal standards (Ameri and Wilkerson, 2008). Other methods, such as refractometry, zinc sulfite turbidity test, sodium sulfate turbidity test, serum γ-glutamyl transferase activity, whole blood glutaraldehyde coagulation test, and ELISA, have been used to identify calves with FTPI with varying degrees of accuracy (Tyler et al., 1996b; Parish et al., 1997; Weaver et al., 2000). Despite these options, there is a need for an accurate, simple, rapid, and cost-effective method for quantification of IgG concentrations in bovine serum and for diagnosis of FTPI in dairy calves, which may be readily translated to the farm, clinic or small laboratory setting.

Transmission IR spectroscopy, in combination with partial least squares (PLS) regression, has been used to measure total serum protein, glucose, albumin, triglyceride, cholesterol, and urea in human serum samples (Ward et al., 1989; Budinova et al., 1997; Shaw et al., 1998). In veterinary applications, it is also widely used for nutrient analysis of milk (Rutten et al., 2011a; Rutten et al., 2011b), and screening of dairy cows for metabolic diseases such as ketosis (Hansen, 1999). Technical difficulties commonly encountered with transmission technique can make routine collection of high-quality spectra in the field situation difficult (Oberg and Fink, 1998). These difficulties include practical issues associated with filling and cleaning short-pathlength cells (for spectroscopy of liquid samples), uncertainties in optical pathlength (for spectroscopy of dried films), and the time required for sample submission and preparation (Sun, 2009; Smith, 2011). In contrast to transmission IR spectroscopy, attenuated total reflectance infrared (ATR) spectroscopy by its nature does not have issues associated with optical pathlength and/or sample thickness. The measurement is simple and fast, and requires

little or no sample preparation (Fahrenfort, 1961). For these reasons, ATR has emerged as one of the most commonly used IR spectroscopic sampling techniques, with applications including various exploratory studies addressing clinical diagnostic problems in human and veterinary medicine (Ellis et al., 2002; Baldauf et al., 2006; Gupta et al., 2006). Most recently robust, small footprint ATR equipment has been manufactured which is ideally suited for field use on the farm, veterinary clinic or small laboratory (Seigneur et al., 2015).

The objectives of this study were to investigate the potential use of ATR, in combination with multivariate data analysis, for the rapid quantification of IgG concentrations in bovine serum, and for the diagnosis of FTPI in dairy calves. This study also investigated the effects of different spectral data pre-processing techniques on model performance and predictive accuracy.

3.3 MATERIALS AND METHODS

3.3.1 Serum samples

Serum samples (n = 250) from Holstein-Friesian calves and adult dairy cows were used. Calf samples (n = 208, including eight prior to colostrum ingestion) had previously been collected for companion studies (Fecteau et al., 1997a; Fecteau et al., 1997b), and adult dairy cow samples (n = 42) had been collected for the Maritime Quality Milk Laboratory (Charlottetown, Prince Edward Island, Canada). Samples were stored at -80°C at the University of Prince Edward Island before use. All samples were tested at the

same time, using a radial immunodiffusion (RID) assay to quantify the serum IgG level and ATR spectroscopy to acquire the IR spectrum for each sample.

3.3.2 RID assay for IgG

A commercial RID assay (Bovine IgG RID Kit, Triple J Farms; Bellingham, WA) was used as the reference method for determining serum IgG concentrations. Serum samples were thawed at room temperature and then vortexed for 10 s. Subsequently, IgG was measured using the bovine RID assay with a working range of 196 to 2,748 mg/dL. For the eight samples that were collected prior to colostrum ingestion, IgG was quantified using the Bovine Ultra Low Level IgG RID Kit with a working range of 10 to 100 mg/dL. Each RID assay was performed according to the manufacturer's instructions using 5 μL of undiluted serum in each well. The diameters of the precipitating zones were measured after 18-24 h by the same individual, using a handheld caliper. Each sample and manufacturer's standards were tested in replicates of five, with one replicate per RID plate, and the mean values were calculated. The assay standards were used to construct a calibration curve that was used to determine the IgG concentrations of the study serum samples.

3.3.3 ATR assay for IgG

IR spectra were acquired using a customized 3-bounce attenuated total reflectance mid-infrared spectrometer (Cary 630 IR spectrometer, 3B Diamond ATR Module ZnSe element, Agilent Technologies, Dansbury, Connecticut). Thawed serum samples were diluted (1:1) with deionized sterile water and vortexed at a maximum of 2700 rpm for 10

s to homogenize the samples. Following dilution, 5 μL aliquots were evenly spread onto the ATR element of the optics module of the spectrometer and dried by a stream of air from a domestic hair dryer. The sample was completely dried within 3-4 min and formed a thin film on the optical element.

Spectra were collected over the wavenumber range of 4000 - 650 cm^{-1} with a nominal resolution of 8 cm^{-1}. For each spectrum, 32 scans were co-added to increase the signal-to-noise ratio. Before each measurement, the stage of the optics module of the spectrometer was cleaned with 100% ethanol and allowed to dry, and a new background reading was collected. Each serum sample was tested in replicates of five. A total of 1250 (250 x 5) ATR spectra were collected and saved in GRAMS spectrum (SPC) format (GRAMS/AI version 7.02, Thermo Fisher Scientific), and then converted into PRN (printable) formatted data. The PRN format spectral data were imported into MATLAB® (version R2012b, MathWorks, Natick, MA) and further data analysis was performed using scripts written by the authors.

3.3.4 Spectral pre-processing

Several pre-processing methods, including Savitsky–Golay smoothing (2^{nd} order polynomial function with 9 points), first-order and second-order derivative spectra (Savitzky and Golay, 1964), and two different normalization methods (Standard normal variate (SNV) and vector normalization) (Barnes et al., 1989; Barnes et al., 2004) were applied to examine effects, if any, on the calibration models. This was followed by spectrum region selection of the 3700–2600 cm^{-1} and 1800–1300 cm^{-1} wavenumber regions, which exhibited the strongest absorptions in the original spectra. With five

replicate spectra per serum sample, spectrum outlier detection was performed using Dixons Q-test (Dean and Dixon, 1951; Rorabacher, 1991) at each wavenumber. If absorbance values were outliers (95% confidence level) for over 50% of the spectral data points for a given spectrum, that complete spectrum was treated as an outlier and excluded from further analysis. The average of the replicate spectra for each sample (after removal of outliers) was used for subsequent analysis.

3.3.5 Multivariate calibration models development

The 250 serum samples were sorted based on IgG concentrations obtained from the RID assays. Serum samples with IgG concentrations outside of the manufacturer's stated performance range for the RID assay were excluded from further analysis (n = 50; 28 calves; 22 cows). The remaining samples (n = 200) were linked to their corresponding pre-processed IR spectra, and then split into two sets (prediction and calibration sets). The prediction set (n = 67) was identified by ordering all of the serum samples according to their corresponding IgG levels and selecting the spectra of every third serum sample as a member of the prediction set. Thus, the prediction set encompassed the full range of IgG values for use in testing the predictive performance of the calibration models. The remaining 'calibration' set (n = 133) was randomly split into training (n = 67) and validation (n = 66) data sets for model development.

PLS regression was used to develop calibration models to relate spectroscopic features (pre-processed wavenumber bands) to the reference serum RID IgG concentrations. PLS regression was applied first to the training set, with up to 30 PLS factors retained, to develop 30 trial calibration models. Each of these models was

employed to calculate the IgG concentration of each sample in the validation set, and an error estimate (sum of the squares of the differences between RID IgG values and the ATR predicted IgG values) was calculated. This procedure was repeated 10,000 times (including new randomly assigned splits of the calibration data set into new training and validation sets). The root mean squared error for the Monte Carlo cross-validation value (RMMCCV) (Picard and Cook, 1984; Xu and Liang, 2001) was calculated for each of the 30 trial calibration models, and then the optimal number of PLS factors was chosen as the one giving the lowest RMMCCV. Once the number of PLS factors was determined, the training and validation sets were combined (i.e. all the samples in the calibration set) to evaluate a PLS calibration model with the determined number of PLS factors.

3.3.6 Evaluation of the calibration models

The predictive accuracy of each multivariate calibration model was assessed using the independent prediction data set (n = 67). The level of agreement between IgG concentrations measured by RID assay and predicted by ATR assay was first assessed for both the calibration and prediction sets by a scatter plot, Pearson correlation coefficient, and the concordance correlation coefficient. This was followed by a Bland–Altman plot (Altman and Bland, 1983; Bland and Altman, 1995), which was used to examine the differences between RID and ATR assays values for the prediction set, thereby assessing the interchangeability of the two assays.

The precision of both the ATR and RID methods was investigated using the prediction set samples. The mean and standard deviations (SD) were calculated for the five replicates of each serum sample, and from these a modified coefficient of variation

(CV* = CV (1 + (1/4n)); n = 5) was determined (Salkind, 2010). For each sample, and for each analytical method, the CV* was then plotted against the mean IgG concentration.

Finally, the potential utility of the ATR method was further assessed using the ratio of predictive deviation (RPD: the population standard deviation of the RID-determined IgG concentrations, ratioed to the RMSEP for the ATR method), and the range error ratio (RER: the range of the RID-determined IgG concentrations, ratioed to the RMSEP for the ATR method) (Williams and Sobering, 1996). According to this framework, a RPD <2 is considered to be poorly predictive; values between 2.0 and 2.5 are adequate for qualitative evaluation or for screening purposes; values >2.5 (or RER >10) are regarded as acceptable for quantification; and values >3 (or RER >20) suggest that the model is suitable for very accurate quantitative analysis (Williams and Sobering, 1996).

3.3.7 Clinical utility of ATR assay

To evaluate the clinical utility of the ATR assay for the diagnosis of FTPI (serum IgG <1,000 mg/dL) in dairy calves, the test characteristics were calculated using 2x2 tables in prediction and entire data sets. These calculations were performed with Stata version 13.0 statistical software (StataCorp, College Station, TX). Sensitivity (Se) was defined as the proportion of samples with FTPI, as determined by RID, that were classified as positive by the ATR assay. Conversely, specificity (Sp) was defined as the proportion of samples without FTPI that were classified as negative by the ATR assay. Accuracy was defined as the proportion of samples that were correctly classified by the ATR assay.

3.4 RESULTS

3.4.1 RID assay

The IgG concentrations of the serum samples with concentrations within the range of the RID assays (n = 200) ranged from 5.5 to 2,983 mg/dL, with an average and SD of 1,122 and 866 mg/dL, respectively. Separate IgG concentration statistics (mean, SD and range) for the calibration set and the prediction set are summarized in Table 3.1.

3.4.2 ATR spectra

A typical ATR spectrum of bovine serum over wavenumber range of 4000 to 650 cm^{-1} is shown in Figure 3.1 Strong absorption bands at 1650 cm^{-1} and 1550 cm^{-1}, correspond to C=O stretching and N–H bending vibrations respectively, while a broad strong absorption band centered at 3300 cm^{-1} was attributed to N–H stretching vibration (Shaw et al., 1998; Shaw and Mantsch, 2000). The noise in the 2200 – 1900 cm^{-1} range was due to absorptions by the diamond coating on the ZnSe ATR element. The spectral region at 4000 – 3800 cm^{-1} was a true baseline, free of appreciable sample absorptions, and as such was highly reproducible for all spectra.

3.4.3 Multivariate calibration model

Trial PLS models were built to compare several pre-processing methods. The optimized results were obtained using data from the smoothed spectra with a 9 point Savitzky-Golay filter and standard normal variate (SNV) scaling (Table 3.2). The

optimum number of PLS factors for this model was 14 (Figure 3.2), based on the lowest IgG RMMCCV (332 mg/dL). Figure 3.2 also shows root mean squared error of calibration (RMSEC) and root mean squared error of prediction (RMSEP) plotted against the number of PLS factors.

3.4.4 Calibration model validation

Figure 3.3 shows a scatter plot that indicates the level of agreement between IgG concentrations measured by the RID assay and those predicted by ATR spectroscopy for the calibration and prediction sets. The plots for the prediction set showed dispersions similar to those for the calibration set, with no significant over-fitting or under-fitting observed, indicating that a robust calibration model was developed. The Pearson correlation coefficients (r) for the calibration and prediction sets were 0.97 and 0.93, respectively. The concordance correlation for the calibration set was 0.97 and for the prediction set 0.93.

The Bland-Altman plot (Figure 3.4) revealed that the mean value of the differences between concentrations obtained by the ATR and RID was -30 mg/dL, which approached zero, indicating no obvious bias between the ATR and RID methods. The 95% confidence interval ranged from -670 mg/dL to 611 mg/dL, which was relatively small in comparison with the range of IgG concentrations (~3,000 mg/dL) obtained from the RID assay. Gauges of precision for the ATR and RID analytical methods are summarized graphically in Figures 3.5A and 3.5B. The mean CV* for ATR analyses was 20%, and the mean CV* for RID analyses was 8.6%. The RPD and RER values were estimated at 2.7 and 9.1, respectively.

3.4.5 ATR sensitivity and specificity for detection of FTPI

The test characteristics of ATR assay were determined for diagnosis of FTPI (serum IgG <1,000 mg/dL) in the prediction and entire data sets. The sensitivity, specificity, and accuracy are shown in Table 3.3. Within the entire data set, the number of samples that had IgG concentrations from the RID assay of <1,000 mg/dL was 102 of 200 samples, resulting in a true FTPI prevalence of 51%. The number of samples that had IgG concentrations <1,000 mg/dL from ATR assay was 94 of 200 samples, resulting in an apparent FTPI prevalence of 47%. There were no false positives and 8 false negatives identified (Table 3.3).

3.5 DISCUSSION

In this study, the authors successfully developed ATR assay as a tool for the rapid measurement of IgG concentrations in bovine serum. The analytical method development required the use of a large calibration data set with a wide range of IgG concentrations to develop a multivariate regression model that could be then applied to determine the IgG concentrations of new serum samples (Murray, 1986).

The performance of the analytical method depends on the spectral pre-processing approaches chosen (Zeaiter et al., 2005). To seek the optimal choice, different pre-processing strategies were evaluated, and the most accurate was selected according to model performance metrics (i.e. lowest RMMCCV and *r* closet to 1) and confirmed by its high predictive accuracy (i.e. low RMSEP, high RPD and RER values) (Williams and

Sobering, 1996). Regardless of the normalization method applied to PLS analysis, spectral smoothing was universally beneficial (Table 3.2). In contrast to other related studies (Elsohaby et al., 2014; Zhang et al., 2013) the authors found that spectral derivation provided no improvement.

The ATR assay showed higher Pearson correlation and concordance coefficients than have been reported for previous transmission IR spectroscopy-based serum IgG assays for bovine (Elsohaby et al., 2014), equine serum and plasma (Riley et al., 2007; Hou et al., 2014), and alpaca serum (Burns et al., 2014). Agreement between the ATR and RID assays was poorer at high IgG concentrations than at low IgG concentrations (Figure 3.3). This may be attributed to the large number of serum samples with IgG concentrations below 1,000 mg/dL (102 out of 200). As a result, the calibration model development was weighted towards low IgG concentrations, which are particularly more important for the diagnosis of FTPI in farm animals (Tyler et al., 1996a; Godden, 2008). Similar findings have been observed for transmission IR spectroscopy-based serum IgG assays for bovine serum (Elsohaby et al., 2014) and IR-based assays for other species (Burns et al., 2014; Hou et al., 2014).

The precision of the ATR analytical method was found to be lower than that of the reference RID assay, as previously observed also for a transmission IR spectroscopy-based assay (Elsohaby et al., 2014). The relatively large CV* for the ATR assay typically occurs because the samples in the prediction set are not involved in the optimization of the calibration model (to ensure that the model performance is not overly optimistic). Nevertheless, given the conservative nature of this estimate of precision, the CV* of the IgG concentrations from the prediction samples lies within the acceptable

range, according to the quality control standards of the US Food and Drug Administration Agency (US Department of Health and Human Services, 2001).

In anticipation of its application in the field, the ATR-based IgG assay was evaluated for its capacity to diagnose a clinically relevant problem - the occurrence of FTPI - using an IgG concentration cut-off value of 1,000 mg/dL (Godden, 2008). The ATR-based assay showed excellent sensitivity (0.92) and specificity (1.0), with values markedly better than those reported for a previously described transmission IR spectroscopy-based assay (Elsohaby et al., 2014). In comparison with other methods reported to assess FTPI in neonates, these results are equivalent to or better than most published assays (Tyler et al., 1996a; Tyler et al., 1996b; Lee et al., 2008). The 8 false negatives for the ATR method corresponded to samples with RID-determined IgG values between 727 - 886 mg/dL, relatively close to the 1,000 mg/dL diagnostic cut-off. These values indicate only partial FTPI, and thus the possible misdiagnosis of these animals poses a substantially lower risk of morbidity and mortality than would be the case for samples with lower IgG concentrations (Lee et al., 2008; Fecteau et al., 2013). There were no false positives identified by ATR spectroscopy. The very low false positive rate has previously been noted for transmission IR spectroscopy-based assays for camelids (no false positives out of 175 samples) (Burns et al., 2014), and bovine samples (4 false positives out of 200 samples) (Elsohaby et al., 2014).

At present, the RID assay is acknowledged to be the reference standard test for quantification of IgG in bovine serum (McBeath et al., 1971). In practice, measurement of IgG by RID method is time-consuming (18 – 24 h), utilizes reagents, and is expensive. In contrast, the ATR assay described in the current work is performed rapidly (one test

can be completed within 3 – 4 min using 5 µL of the sample) and the sample can be used with dilution in deionized water, the only required sample preparation step. These attractions, combined with practical advantages associated with compact, portable ATR spectrometers (Sun, 2009; Smith, 2011), suggest the real possibility of using the technique in the field for assessing colostrum management, and ensuring adequate transfer of passive immunity to neonatal calves.

3.6 CONCLUSIONS

ATR in combination with multivariate data analysis is a feasible alternative for the rapid quantification of IgG concentrations in bovine serum and has the potential to effectively assess FTPI in neonatal calves. Testing of different pre-processing approaches revealed that spectral smoothing (without spectral derivation) significantly improved analytical performance and accuracy as compared to otherwise identical methods with no spectral smoothing.

3.7 REFERENCES

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Table 3.1. Descriptive statistics of immunoglobulin G (IgG) concentrations measured by the reference method of radial immunodiffusion (RID) assay in the calibration and prediction data sets.

Item	Calibration set				Prediction set			
	N	Mean	SD	range	N	Mean	SD	range
RID IgG	133	1117	864	2975	67	1132	876	2977

N: number of samples in the calibration and prediction sets; SD: standard deviation (mg/dL); range: difference (highest minus lowest) of IgG concentration (mg/dL)

Table 3.2. Comparison of calibration models and prediction results of the immunoglobulin G (IgG) concentration of 200 bovine serum samples, obtained using different pre-processing approaches for infrared spectra

Pre-processing	PLS factors	RMMCCV (mg/dL)	Calibration (n = 133)		Prediction (n = 67)			
			r	RMSEC (mg/dL)	*r*	RMSEP (mg/dL)	RPD	RER
Smoothing (9 points)	14	335	0.97	364	0.92	340	2.6	8.8
Smoothing + normalization (SNV)	14	332	0.97	359	0.93	326	2.7	9.1
Smoothing + vector normalization	14	336	.097	367	0.93	331	2.7	9
1st derivatives (9 points)	6	341	0.95	370	0.91	373	2.4	8
1st derivatives + normalization (SNV)	6	338	0.95	355	0.91	362	2.4	8.2
1st derivatives + vector normalization	6	340	0.96	357	0.91	362	2.4	8.2
2nd derivatives (9 points)	5	363	0.97	382	0.88	424	2.1	7
2nd derivatives + normalization (SNV)	5	358	0.97	376	0.87	429	2	7
2nd derivatives + Vector normalization	6	356	0.97	376	0.89	409	2.1	7.3

PLS: Partial least squares; RMMCCV: Root mean squared error of the Monte Carlo cross-validation value; *r:* Pearson correlation coefficient; RMSEC: Root mean squared error of calibration; RMSEP: Root mean squared error of prediction; RPD (ratio of predictive deviation): SD divided by RMSEP; RER (range error ratio): Range divided by RMSEP; SNV: Standard normal variate.

Table 3.3. Sensitivity, specificity, and accuracy of ATR-based IgG assay as a diagnostic test method to determine failure of transfer of passive immunity (FTPI) in the prediction (n = 67) and entire (n = 200) data sets

Data sets	N	Test characteristics						
		True positives	False positives	True negatives	False negatives	Se	Sp	Accuracy
Prediction	67	30	0	33	4	88%	100%	94%
All data	200	94	0	98	8	92%	100%	96%

N: Number of samples; Se: Sensitivity; Sp: Specificity.

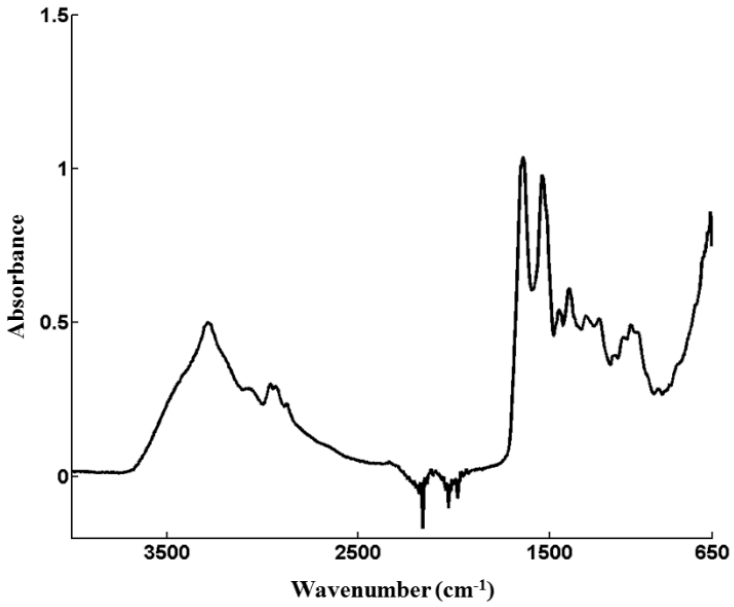

Figure 3.1. A representive raw spectrum of a bovine serum sample over the spectral range of 4000 – 650 cm^{-1} obtained by attenuated total reflectance infrared spectroscopy.

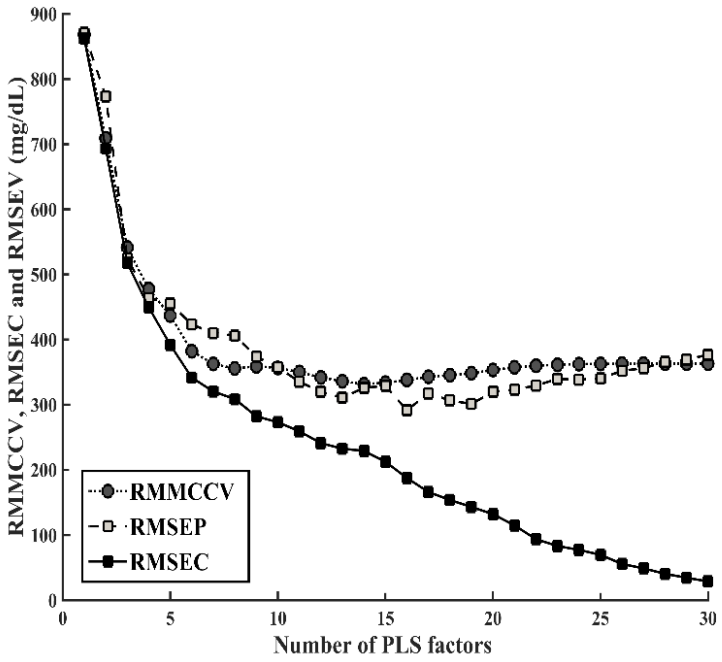

Figure 3.2. Plots of RMMCCV and RMSEC for the calibration data set, and RMSEP for the prediction data set. The optimum number of PLS factors was determined to be 14, based on the lowest RMMCCV. RMMCCV: Root mean squared error in the Monte Carlo cross-validation value; RMSEC: Root mean squared error of calibration; RMSEP: Root mean squared error of prediction; PLS: Partial least squares.

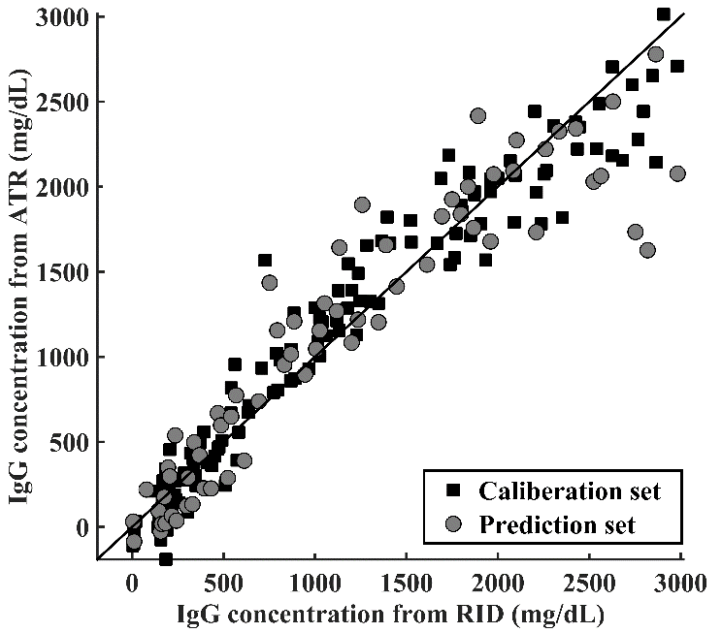

Figure 3.3. Scatter plots comparing immunoglobulin G (IgG) concentrations measured by the radial immunodiffusion (RID) assay to those predicated by the ATR-IR spectroscopy-based assay. The correlation coefficients (*r*) were 0.97 and 0.93 for the calibration and prediction data sets, respectively. The squares donate the samples from the calibration set, and the circles indicate the samples from the prediction set. The two assays are considered comparable if data points distribute closely around the reference line.

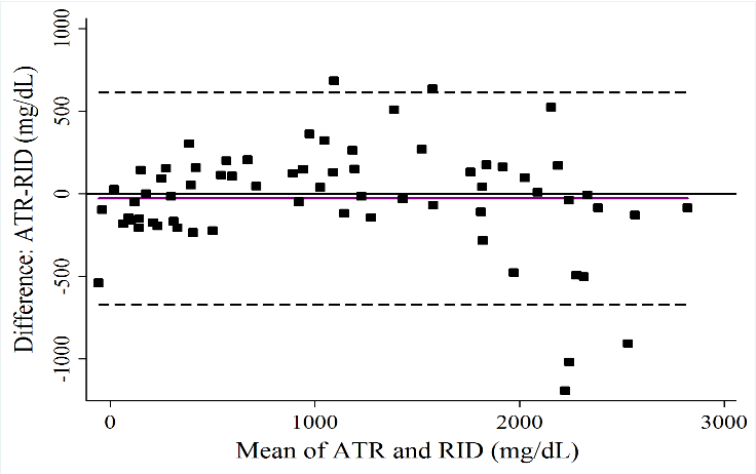

Figure 3.4. Bland–Altman plot of the average immunoglobulin G (IgG) concentrations measured by RID and ATR-IR methods (x-axis) against the difference in IgG concentrations between the two methods (y-axis) for the prediction set samples (n = 67). The dashed lines represent the 95% confidence limits of agreement (–670 to 611 mg/dL), and the solid line represents the mean difference between ATR-IR and RID assays (-30 mg/dL), indicating no appreciable systematic difference between the two methods.

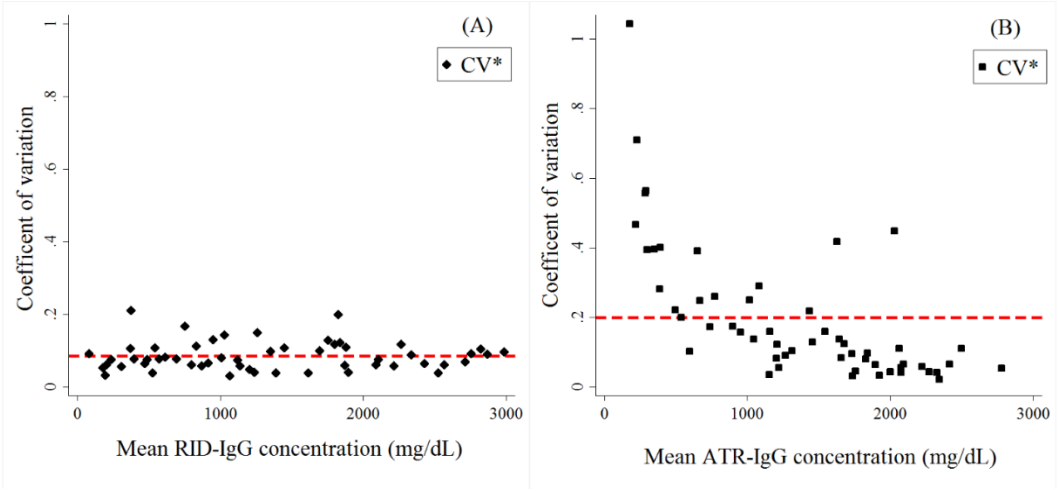

Figure 3.5. Adjusted coefficient of variance (CV) plots for (A) the RID method and (B) the ATR-IR method. The dashed line represent the mean CV for RID assay (0.086, or 8.6%) and ATR-IR spectroscopy (0.2, or 20%).

CHAPTER 4

QUANTIFICATION OF BOVINE IMMUNOGLOBULIN G USING TRANSMISSION AND ATTENUATED TOTAL REFLECTANCE INFRARED SPECTROSCOPY

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4.1 ABSTRACT

In this study, we evaluated and compared the performance of transmission and attenuated total reflectance (ATR) infrared spectroscopic methods (in combination with quantitative algorithms previously developed using partial least squares regression) for the rapid measurement of bovine serum IgG concentration, and diagnosis of failure of transfer of passive immunity (FTPI) in dairy calves. Serum samples (n = 200) were collected from Holstein calves from 1 to 11 days of age. Serum IgG concentrations were measured by the reference method of radial immunodiffusion (RID) assay, transmission-IR and ATR-IR spectroscopy-based assays. The mean IgG concentration measured by RID was 1,722 mg/dL (SD ± 960). The mean of IgG concentrations predicated by transmission-IR and ATR-IR spectroscopy methods were 1,560 mg/dL (SD ± 815) and 1,594 mg/dL (SD ± 866), respectively. RID IgG concentrations were positively correlated with IgG levels predicted by transmission-IR (r = 0.94) and ATR-IR (r = 0.92). The correlation between two IR spectroscopic methods was 0.94. Using IgG concentration <1,000 mg/dL as cut-point for FTPI positive cases, the overall percent of agreement between transmission-IR and ATR-IR methods was 94%, with a corresponding kappa-value of 0.84. The Se, Sp, PPV, NPV and accuracy for identifying FTPI by transmission-IR were 0.87, 0.97, 0.91, 0.95 and 0.94, respectively. Corresponding values for ATR-IR were 0.87, 0.95, 0.86, 0.95 and 0.93, respectively. In conclusion, both transmission-IR and ATR-IR spectroscopic approaches can be used for rapid quantification of IgG level in neonatal bovine serum and for diagnosis of FTPI in dairy calves.

4.2 INTRODUCTION

Adequate passive transfer of maternal immunoglobulin (Ig) via colostrum is critical for the optimal health and performance of dairy calves (Chigerwe et al., 2008). Failure of calves to ingest and/or absorb sufficient amounts of colostral Ig (<1,000 mg/dL) results in a condition known as failure of transfer of passive immunity (FTPI) (Godden, 2008). There is a recognized association between FTPI and calf morbidity, mortality and reduced daily gain in the first few months of life (Robison et al., 1988; Donovan et al., 1998; Virtala et al., 1999). Moreover, FTPI reduces long-term productivity, decreases milk yield, and increases culling rates during the first lactation of dairy heifers (DeNise et al., 1989; Heinrichs and Heinrichs, 2011). As a consequence of FTPI in affected calves, dairy producers experience increased production costs and reduced profitability. It is vitally important for dairy producers and veterinarians to ensure that dairy calves receive and absorb a sufficient quantity of good quality colostrum within the first hours of life (Morin et al., 1997; Jaster, 2005).

Passive transfer of immunity in calves may be identified by several methods. The radial immunodiffusion (RID) assay, enzyme-linked immunosorbent assay (ELISA) and automated turbidimeteric immunoassay directly measure serum IgG concentrations (Filteau et al., 2003; Alley et al., 2012). Refractometry, the sodium sulfite turbidity test, zinc sulfate turbidity test, serum gamma-glutamyl transferase activity and whole blood glutaraldehyde coagulation can all be used to estimate serum IgG concentration indirectly (Calloway et al., 2002; McGuirk and Collins, 2004; Wallace et al., 2006). RID is currently considered the reference method for measuring IgG concentration and assessing

FTPI in dairy calves (McBeath et al., 1971). The method is laboratory-based, relatively expensive, and requires a minimum of 18 to 24 hr to obtain the results. Thus, is not practical for routine, on-farm monitoring of the adequacy of transfer of passive immunity (Riley et al., 2007; Bielmann et al., 2010).

Infrared (IR) spectroscopy in combination with multivariate data analysis has emerged as an alternative technique for assessing FTPI in several species and has the advantages of requiring a small sample volume, rapid turnaround time, minimal disposable materials, low-cost, and robust predictions (Shaw et al., 1998; Shaw and Mantsch, 1999; Shaw and Mantsch, 2000). Methods employing IR spectroscopy have been successfully used to determine nutrient contents of milk (Rutten et al., 2011a; Rutten et al., 2011b) colostrum IgG concentration (Rivero et al., 2012) feed composition and the kinetics of nutrient degradation in the rumen (Herrero et al., 1997; Ohlsson et al., 2007). They have also been utilized in screening for metabolic diseases such as ketosis (Hansen, 1999) and foodborne pathogens (Davis and Mauer, 2010). However, there are a number of various IR spectroscopic sampling techniques. Two of the most common techniques are transmission-IR and attenuated total reflectance (ATR-IR) spectroscopy. Transmission-IR spectroscopy has been used for decades in different fields, including chemistry, medicine, biology, and geology (Stuart, 2005). However ATR-IR has the advantage of reduced sample preparation time, and is relatively impervious to variations in sample thickness compared to transmission-IR, making collection of high-quality spectra easier (Oberg and Fink, 1998; Sun, 2009; Smith, 2011). The objective of this study was to investigate the utility of previously built partial least squares (PLS) models for quantifying IgG for a new set of samples and to compare the performance of

transmission-IR and ATR-IR spectroscopic approaches (in combination with multivariate analysis) as the basis to predict IgG levels in bovine serum, and to diagnose FTPI in dairy calves.

4.3 MATERIALS AND METHODS

4.3.1 Serum samples

Blood samples were collected from 203 Holstein calves between 1 and 11 days of age. Calf samples came from 5 different commercial dairy farms in Prince Edward Island and one in Nova Scotia between June and October, 2013. Whole blood was collected by jugular venipuncture, using a 20-gauge, 1-inch hypodermic needle, into a sterile, plastic, vacutainer tube without anticoagulant (BD Vacutainer Precision Glide, Becton Dickinson Co., Franklin Lakes, NJ). Samples were transported in a cooler at 4 °C to the Maritime Quality Milk Laboratory, University of Prince Edward Island (UPEI). Serum was separated by centrifugation at 1,500 g for 10 min within 5 hr of collection. Serum from Nova Scotia samples was separated by similar centrifugation methods at a local veterinary clinic and then frozen at -20 °C for batch transport to UPEI. Serum samples were divided into 3 aliquots and stored at -80 °C. This study was conducted in accordance with the Canadian Council on Animal Care (CCAC) guidelines (Care, Canadian Council On Animal, 2009) under a protocol approved by the Animal Care Committee at UPEI.

4.3.2 Transmission-IR spectroscopy

The sampling and data acquisition protocols matched those used previously for developing the transmission-IR spectroscopy bovine IgG assay (Elsohaby et al., 2014). Serum samples were thawed at room temperature (20 – 24 °C) and then vortexed for 10 s. Samples were then diluted (1:1) with deionized sterile water and vortexed at a maximum of 2,700 rpm for 10 s. After dilution, six replicates were made for each serum sample by evenly spreading 10 µl aliquots of diluted sample onto 5 mm diameter wells within an adhesive-masked, 96-well silicon microplate (Riley et al., 2007). An empty well served as the background reference for each microplate. The loaded microplates were allowed to dry at room temperature (20–24 °C) for 2 hr, resulting in thin dried films. For collection of transmission-IR spectra, the microplates were inserted into a multisampler (HTS-XT Autosampler, Bruker Optics Ltd, UK) interfaced with a Bruker IR spectrometer (Tensor 37, Bruker Optics Ltd, UK) equipped with a deuterium tryglycine sulfate detector and controlled by proprietary software (OPUS version 6.5, Bruker Optics Ltd, UK). A total of 1,218 (203 × 6) spectra were collected over the wavenumber range between 4,000 and 400 cm^{-1} with a nominal resolution of 4 cm^{-1} with 512 scans collected for data acquisition.

4.3.3 ATR-IR spectroscopy

The sampling and data acquisition protocols matched those used previously for developing the ATR-IR spectroscopy bovine IgG assay (Elsohaby et al., 2015). The ATR-IR spectra of serum samples were acquired using a customized 3-bounce attenuated total reflectance mid-infrared spectrometer (Cary 630 FTIR spectrometer, 3B Diamond

ATR Module ZnSe element, Agilent Technologies, Dansbury, Connecticut). Prior to applying each sample a background spectra was collected over the wavenumber range of 4,000 - 650 cm^{-1} with a resolution of 8 cm^{-1} and 32 scans were co-added for each spectrum. Thawed serum samples were diluted (1:1) with deionized sterile water and vortexed at a maximum of 2,700 rpm for 10 s to homogenize the samples. Following dilution, five replicates were made for each sample by evenly spreading of 5 µl aliquots of diluted sample onto the sample stage and dried by air using a domestic hair dryer. Samples were completely dried within 3-4 min, forming a thin film on the stage. Before each successive test, the stage of the optics module of the spectrometer was cleaned with 100% ethanol, and a new background spectrum was collected. A total of 1,015 (203 x 5) ATR-IR spectra were collected and saved in spectrum (SPC) format.

4.3.4 Spectral pre-processing

Spectra were collected and converted into a printable format (PRN) using GRAMS software (GRAMS/AI version 7.02, Thermo Fisher Scientific nc., Waltham, MA). The PRN format spectral data were imported into MATLAB version R2012b (MathWorks, Natick, MA) and further data analysis was performed using scripts written by the authors. Spectra collected from both spectroscopies were pre-processed using the same techniques used for partial least squares (PLS) model building (Elsohaby et al., 2014; Elsohaby et al., 2015). Spectra were pre-processed using the Savitzky–Golay method (second order polynomial function with 9 points smoothing) to generate 1st order derivative and 2nd order derivative spectra for transmission and ATR-IR spectroscopic methods, respectively (Savitzky and Golay, 1964). The derivative spectra then were

normalized using a vector normalization procedure to reduce the effect of light scattering, followed by spectral sub-region selection at 3,700 – 2,600 cm^{-1} and 1,800 - 1,300 cm^{-1} wavenumber regions, which exhibited the strongest absorptions in the original spectra. Subsequently, spectrum outlier detection was performed using Dixons Q-test (Dean and Dixon, 1951; Rorabacher, 1991) at each wavenumber. Spectra were designated as outliers if more than 50% of absorbance values were outside the 95% confidence level, and excluded from further analysis. The remaining spectrum of each sample (after removal of outlier spectra if applicable) was used for subsequent analysis.

4.3.5 Prediction of IgG

Previously developed PLS models built for prediction of IgG concentration from transmission IR spectra (Elsohaby et al., 2014) and ATR-IR spectra (Elsohaby et al., 2015) were used to predict serum IgG concentrations in these 203 calf samples. These PLS models were built using 200 serum samples collected from calves and adult cows (Elsohaby et al., 2014). The IgG concentration was predicted from each spectrum, and subsequently the IgG concentration for each calf serum sample was calculated as the average of replicate IgG values.

4.3.6 RID assay

A commercial RID assay (Bovine IgG RID Kit, Triple J Farms; Bellingham, WA) was used as the reference method for determining calf IgG serum concentrations. The RID assay was performed according to manufacturer's instructions, using 5µl of undiluted serum sample in each well. Diameters of precipitated rings were measured after

18 – 24 hr of incubation at room temperature, using a handheld calliper. Each of the samples and assay standards were tested in replicates of five. The average IgG values of the replicates of the assay standards were used to build a calibration curve that was subsequently used to determine IgG concentrations for the serum samples. The final IgG concentration for each sample was determined by calculating the average of the five replicates. Serum samples with IgG concentrations greater than the manufacturer's stated performance range for the assay (>3,000 mg/dL) were diluted (50:50) with deionized sterile water and retested. Diluted serum samples still with IgG concentrations outside the performance range for the assays, as determined by the manufacturer, were excluded from further analysis (n = 3).

4.3.7 Statistical analysis

Descriptive statistics for the results of RID, transmission-IR and ATR-IR spectroscopic approaches were calculated. The IgG concentrations for each sample as predicted by transmission-IR and ATR-IR spectroscopy were plotted against each other, and against the IgG concentrations measured by the reference RID method. From these plots, Pearson's correlation coefficients (r) and concordance correlation coefficients were determined. A Bland–Altman plot was used to assess the interchangeability of the two IR spectroscopic methods and the RID assay by testing the difference between values as determined by these methods. The level of agreement between the IgG values for transmission-IR and ATR-IR spectroscopic methods was assessed using McNemar's test (Lachenbruch and Lynch, 1998) for paired data to check for bias, followed by calculation of the kappa statistic. The precision of the RID, transmission-IR and ATR-IR based

analytical methods was investigated. The mean and standard deviations (SD) were calculated for the replicates of each serum sample, and from these a modified coefficient of variation (CV* = CV (1 + (1/4n)); n = 5 for RID and ATR-IR assays, n = 6 for transmission-IR assay) was determined (Salkind, 2010). For comparison, the CV* of each sample tested by RID, transmission-IR and ATR-IR spectroscopic methods was calculated and plotted against the mean IgG concentration. The diagnostic test characteristics (sensitivity, specificity, predictive values and accuracy) were calculated to evaluate the clinical applicability of transmission-IR and ATR-IR methods for the diagnosis of FTPI, using IgG concentrations of <1,000 mg/dL (as measured by RID test) as FTPI positive cases (STATA 13 software, Stata Corp., College Station, TX).

4.4 RESULTS

4.4.1 Descriptive statistics

Descriptive statistics for IgG concentrations measured by the reference RID assay and predicted by transmission-IR and ATR-IR spectroscopy-based assays for samples within the RID reference range (n = 200) are shown in Table 4.1. The frequency distribution of the RID IgG concentrations was skewed to the right (Figure 4.1). However, distribution of IgG concentrations predicated by transmission-IR and ATR-IR spectroscopy-based assays were approximately normally distributed (Figure 4.1).

4.4.2 Agreement between RID and IR spectroscopic methods

Correlations between IgG concentrations measured by the reference RID assay and predicted by both IR spectroscopy-based assays were determined using correlation scatterplots (Figure 4.2). The RID IgG concentrations were positively correlated with those predicted by transmission-IR (r = 0.94, Figure 4.2A) and ATR-IR (r = 0.92, Figure 4.2B) spectroscopy. Similarly, the IgG concentrations predicted by transmission-IR and ATR-IR spectroscopy-based assays were positively correlated (r = 0.94, Figure 4.2C). The concordance correlations between values for RID and both IR spectroscopic methods was 0.91.

The Bland-Altman plot (Figure 4.3) revealed that the mean value of the difference between IgG concentrations provided by the RID and the transmission-IR method was -162 mg/dL (Figure 4.3A), and between RID the ATR-IR method -128 mg/dL for (Figure 4.3B), respectively, indicating no obvious bias between these methods. The 95% confidence interval for transmission-IR ranged from (-838 to 515 mg/dL) and for ATR-IR ranged from (-867 to 710 mg/dL).

The agreement between the two IR spectroscopic methods and the RID assay for assessment of FTPI is presented in Table 4.2. The overall percent of agreement between transmission-IR and ATR-IR was 94%, with a corresponding kappa-value of 0.84. The McNemar's test for the sensitivity and specificity comparisons, showed no significant difference (P >0.05) in the proportion of calves classified as having FTPI by either IR spectroscopic methods.

4.4.3 Precision of RID and IR spectroscopic methods

Figure 4.4 shows the plots of CV* for the RID, transmission-IR and ATR-IR methods. There was no obvious correlation with the IgG concentrations and CV* for the RID method (Figure 4.4A), but the CV* for both transmission-IR (Figure 4.4B) and ATR-IR (Figure 4.4C) methods decreased with increasing IgG concentrations. The mean CV* for RID, transmission-IR and ATR-IR methods were 8.3%, 11%, and 28%, respectively.

4.4.4 Test characteristics of IR spectroscopic methods

The test characteristics of transmission-IR and ATR-IR methods for assessment of FTPI in 200 dairy calves are shown in Table 4.3. Fifty-five samples had IgG concentrations <1,000 mg/dL according to the RID method, resulting in a true FTPI prevalence of 27.5%. The number of samples that had IgG concentrations <1,000 mg/dL according to the transmission-IR (n = 53) and ATR-IR (n = 56) methods resulted in an apparent FTPI prevalence of 26.5% and 28% respectively.

For the transmission-IR method, 5 and 7 samples were misclassified as false positives and false negatives, respectively. These samples corresponded to samples with RID IgG values between 1,014 – 1,359 mg/dL and 793 – 984 mg/dL, respectively. For the ATR-IR method, 8 samples were misclassified as false positive with RID IgG values between 1,022 – 1,260 mg/dL, and 7 samples were misclassified as false negative with RID IgG values between 793 – 984 mg/dL.

4.5 DISCUSSION

These results confirmed that IR spectroscopy, in combination with a quantification algorithm developed using multivariate data analysis, is suitable for the quantification of IgG in bovine serum and diagnosis of FTPI in dairy calves. The two PLS models previously developed as the basis for analytical methods based upon transmission-IR and ATR-IR spectroscopic measurements were validated by their ability to accurately quantify IgG concentrations in a completely independent set of serum samples. It is worth mentioning that the average IgG concentration for this new set of serum samples was higher (Table 4.1) than the corresponding average of IgG concentrations used for the original PLS model development.(Elsohaby et al., 2014) The prevalence (27.5%) of calves with IgG concentration <1,000 mg/dL was correspondingly lower in this data set than that used for the original model development (prevalence 51%), and falls within the range of FTPI prevalence (19 to 40%) reported by the National Animal Health Monitoring System (NAHMS) and a more recent Canadian study (Dairy, 2007; Trotz-Williams et al., 2008).

The correlation coefficients plots for the IgG concentrations predicted by both transmission-IR and ATR-IR spectroscopy-based assays compared to the RID determinations were similar that determined between the two IR methods (Figure 4.2). In comparison, these values are higher than those reported previously for counterpart transmission-IR-based assays for equines IgG (Riley et al., 2007; Hou et al., 2014) and camelid IgG levels, (Burns et al., 2014) and for the ATR-IR method described for canine IgG concentrations (Seigneur et al., 2015). Furthermore, the correlation coefficients

between IgG concentrations predicted by both IR spectroscopic methods and the reference RID IgG assay were higher than that reported for refractometry (Morrill et al., 2013; Deelen et al., 2014) similar to that of near infrared spectroscopy (Zhang et al., 2013) but lower than the value reported for automated turbidimetric immunoassay (Alley et al., 2012).

The level of agreement between results from the two IR spectroscopic methods and the RID assay for detecting FPTI was high, as assessed using kappa statistics (Table 4.2). Similarly, the ATR-IR spectroscopy showed good agreement (94%) with the transmission-IR spectroscopy. This indicates that the two spectroscopic methods demonstrate a high level of agreement on the classification of calves with and without FTPI. The McNemar's test showed a non-significant difference (P >0.05) between proportions of calves classified as having FTPI by the IR methods, indicating that the two spectroscopic assays performed similarly for FTPI assessment in calves. However, transmission-IR showed higher precision than ATR-IR (Figure 4.4). The same results were reported during PLS model building for IgG concentrations of bovine (Elsohaby et al., 2014) equine (Hou et al., 2014) camelid (Burns et al., 2014) and canine samples (Seigneur et al., 2015). The lower precision of ATR-IR method is likely to be associated with the variability in sample dryness for each replicate, and to the low number of ATR-IR scans (32 scans), as compared to transmission-IR method (512 scans) (Sun, 2009; Smith, 2011).

With respect to the assessment of dairy calves for FTPI, both IR-based analytical methods showed excellent specificity and good sensitivity, equivalent to or better than most methods previously reported to assess dairy calves for FTPI (Tyler et al., 1996a;

Tyler et al., 1996b; Lee et al., 2008). Both methods correctly diagnosed the majority of FTPI cases, and the misclassified samples had RID IgG concentrations very close to the diagnostic cut-off of 1,000 mg/dL. This suggests that the likelihood of consequential misdiagnosis is small; the false negative samples correspond to only partial FTPI (IgG value as low as 800 mg/dL) with minimal risk of calf morbidity and mortality (Perino et al., 1993; Lee et al., 2008; Fecteau et al., 2013).

The PPV and NPV values vary with both test performance and the population FTPI prevalence (Riley et al., 2007). In North America, the reported prevalence of FTPI in dairy calves ranges from 19% to 40% (Nocek et al., 1984; Dairy, 2007; Trotz-Williams et al., 2008). When the proportion of calves with FTPI increases, NPV decreases. Because the sensitivity of both IR spectroscopy-based assays was the same, at true prevalence range of FTPI in North America of 19% to 40%, the NPV can be calculated at 97% and 92%, respectively. From NPV, the positive predictive value of negative test (PPVN = 1 – NPV) (Dohoo et al., 2009) can be evaluated. Thus, the probability of FTPI-positive calves being classified by the IR-based assay as test-negative (would not receive treatment) would be 3% (0.03) and 8% (0.08) for herds with FTPI prevalences of 19% and 40%, respectively. The PPV was numerically higher for the transmission IR-based assay than for the ATR-IR assay. As a result the number of FTPI-negative animals that were misidentified as FTPI-positive (and receive additional care and farm inputs) was slightly higher using the ATR-IR assay as compared to the transmission-IR method. While the transmission-IR based assay had a slightly higher specificity, PPV and precision than the ATR-IR based assay, it is a laboratory–based assay. In contrast, commercially available ATR-IR instrumentation are robust and portable making them

more appropriate for use in field situations (e.g. the Agilent 4500, Agilent, Danbury, Connecticut). This opens the door for dairy producers and veterinarians to confirm adequate transfer of passive immunity to their calves on farms or in veterinary clinical settings with ATR-IR spectroscopy.

4.6 CONCLUSIONS

IR-spectroscopic methods in combination with previously developed PLS model has provided rapid, accurate quantification of bovine serum IgG concentration, and diagnosis of FTPI in a new, independent set of samples. Although the transmission-IR based assay showed relatively minor higher specificity, PPV and precision than the ATR-IR based assay, the ATR-IR based assay has attractive practical advantages for potentially monitoring FTPI on farms or in veterinary clinics.

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Table 4.1. Descriptive statistics of serum immunoglobulin G concentrations as measured by radial immunodiffusion (RID) assay, transmission and attenuated total reflectance (ATR) infrared spectroscopic methods for 200 Holstein dairy calves.

Assay	n	Mean	SD	Min	Median	Max
RID (mg/dL)	200	1722	960	133	1592	4302
Transmission-IR (mg/dL)	200	1560	815	-249	1491	3390
ATR-IR (mg/dL)	200	1594	866	-335	1553	3503

Table 4.2. Level of agreement between the two infrared (IR) spectroscopic methods and radial immunodiffusion (RID) assay for assessment failure of transfer of passive immunity (FTPI = serum IgG < 1,000 mg/dL) in 200 dairy calves using kappa statistics.

Assay	Agreement	kappa	P-value
Transmission-IR vs. RID	0.94	0.85	0.0001
ATR-IR vs. RID	0.93	0.81	0.0001
Transmission vs. ATR-IR	0.94	0.84	0.0001

Table 4.3. Diagnostic test characteristics for transmission-IR and ATR-IR spectroscopic methods* for assessment of failure of transfer of passive immunity (FTPI) in 200 dairy calves, using IgG concentration 1,000 mg/dL as a cut-off value and a true FTPI prevalence of 27.5%

Test	Infrared (IR) spectroscopies	
Characteristics†	Transmission-IR	ATR-IR
True positives	48	48
False positives	5	8
True negatives	140	137
False negatives	7	7
Apparent Prevalence	0.265	0.28
Se (95% CI)	0.87 (0.76 – 0.95)	0.87 (0.76 - 0.95)
Sp (95% CI)	0.97 (0.92 – 0.99)	0.95 (0.89 – 0.98)
PPV (95% CI)	0.91 (0.79 – 0.97)	0.86 (0.74 – 0.94)
NPV (95% CI)	0.95 (0.90 – 0.98)	0.95 (0.90 – 0.98)
Accuracy	0.94	0.93

* Transmission-IR = transmission infrared spectroscopic method, ATR-IR = attenuated total reflectance infrared spectroscopic method

† Se = sensitivity; Sp = specificity; PPV = positive predictive value; NPV = negative predictive value; Accuracy = percentage of samples correctly classified as adequate or failure of transfer of passive immunity.

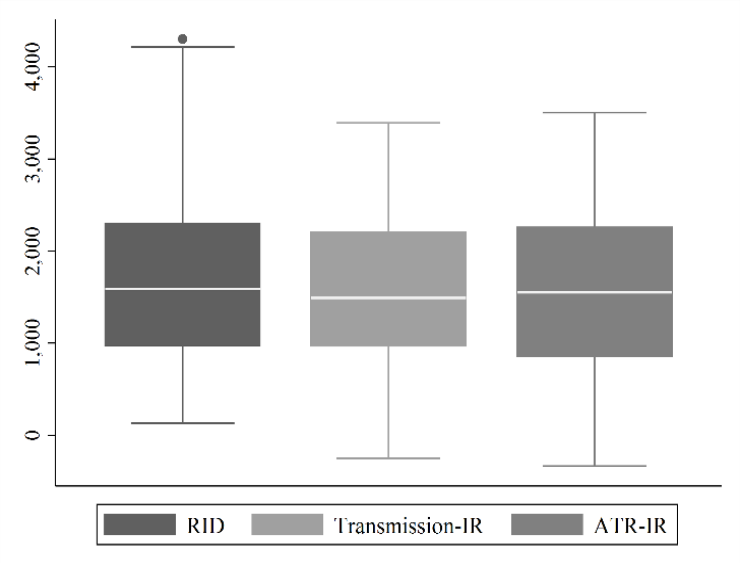

Figure 4.1. Frequency distribution of serum immunoglobulin G concentrations measured by a radial immunodiffusion (RID) assay, and transmission-IR and ATR-IR spectroscopic methods for 200 Holstein dairy calves.

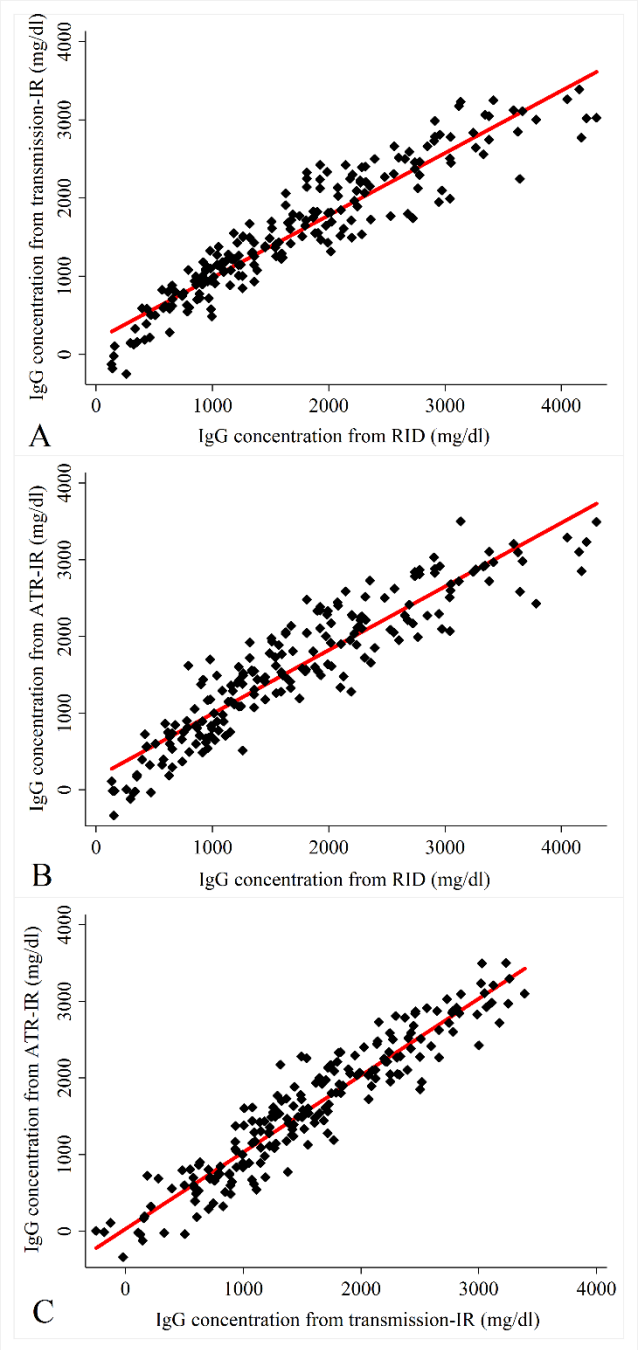

Figure 4.2. Scatter plots comparing (A) serum IgG concentrations obtained by the radial immunodiffusion (RID) assay with IgG concentrations provided by the transmission-IR spectroscopy-based assay (r = 0.94); (B) serum IgG concentrations obtained by the radial immunodiffusion (RID) assay with IgG concentrations provided by the ATR-IR spectroscopy-based assay (r = 0.92); (C) serum IgG concentrations provided by both IR spectroscopic methods (r = 0.94) for 200 Holstein dairy calves.

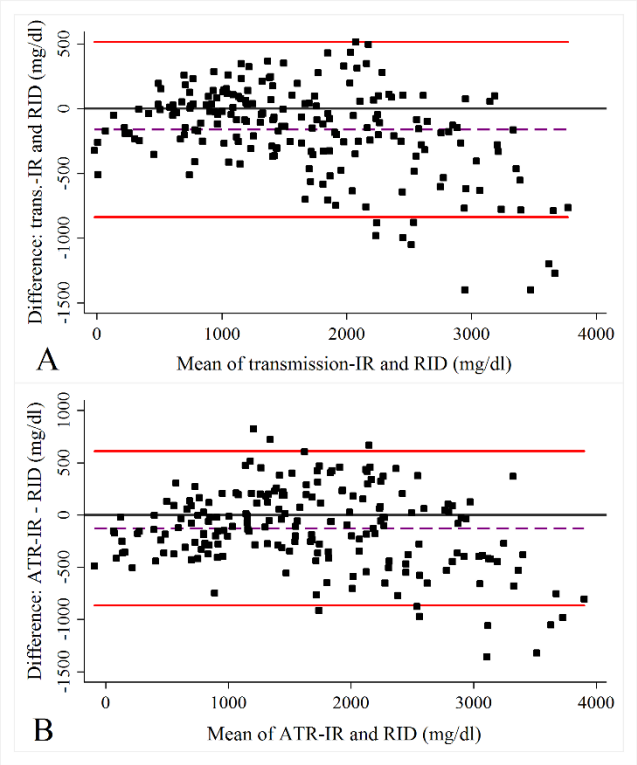

Figure 4.3. Bland-Altman plots of (A) the average immunoglobulin G (IgG) concentrations measured by RID and the transmission-IR spectroscopy-based assay (x-axis) against the difference in IgG concentrations between the two methods (y-axis). The solid lines represent the 95% confidence limits of agreement (-838 to 515 mg/dL), while the dashed line represents the mean difference between transmission-IR and RID methods (-162 mg/dL). (B) The average immunoglobulin G (IgG) concentrations measured by RID and the ATR-IR spectroscopy-based assay (x-axis) against the difference in IgG concentrations between the two methods (y-axis). The solid lines represent the 95% confidence limits of agreement (-867 to 710 mg/dL), while the dashed line represents the mean difference between ATR-IR and RID methods (-128 mg/dL).

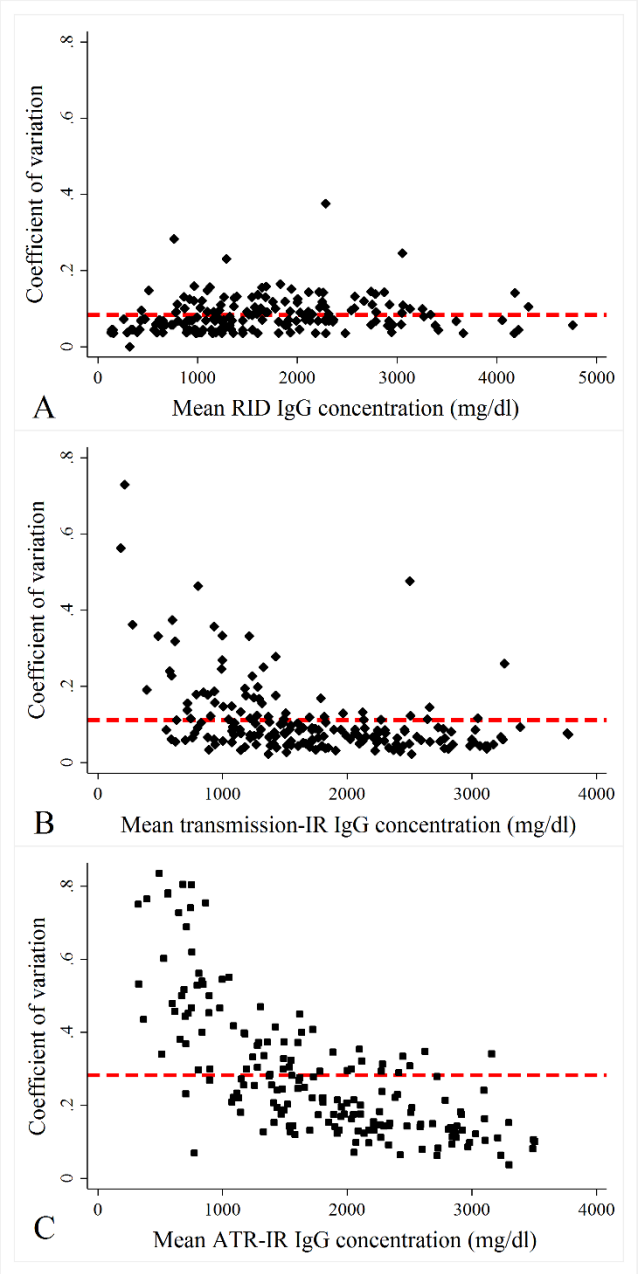

Figure 4.4. Coefficient of variance (CV*) plots for (A) the radial immunodiffusion (RID) method; (B) the transmission-IR spectroscopy-based assay; (C) the ATR-IR spectroscopy-based assay. The dashed lines represent the mean CV* for the RID assay (0.083, or 8.3%; panel "A"), the transmission IR-based assay (0.11, or 11%; panel "B") and the ATR-IR-based assay (0.28, or 28%; panel "C").

CHAPTER 5

A NOVEL METHOD FOR THE QUANTIFICATION OF BOVINE COLOSTRAL IMMUNOGLOBULIN G USING INFRARED SPECTROSCOPY

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5.1 ABSTRACT

Transmission infrared (IR) spectroscopy (in combination with partial least squares (PLS) regression) was used as the basis to develop a new method to quantify IgG in bovine colostrum. Colostrum samples (n = 250) were collected from 7 commercial dairy farms and tested simultaneously by the reference radial immunodiffusion (RID) assay and IR spectroscopy. The colostral IgG concentrations obtained by RID assay were linked to pre-processed spectra and divided into two sets: calibration and test. Partial least squares (PLS) regression was applied to the calibration set (n = 167), and calibration models were developed. The optimal calibration model had 13 PLS factors; lowest root mean squared error for the Monte Carlo cross-validation value (RMMCCV = 16 g/L), and the highest correlation coefficient with RID (r = 0.91). The test set (n = 83) was used to assess the accuracy of the analytical method for blinded samples.

The Pearson and concordance correlations between test set IgG concentrations as determined by the IR assay and the RID assay were 0.91. Based upon the Bland-Altman plot there was no evidence of systematic bias between IR and RID methods. The IR assay showed relatively lower precision than the RID method with a modified coefficient of variation (CV* = 8.9%). The sensitivity, specificity and accuracy of the IR-based assay as a diagnostic test to differentiate between low and high quality colostrum (for the entire data set) were 90%, 92%, and 90%, respectively. Transmission IR spectroscopy is an effective method for quantification of bovine colostral IgG concentration and for assessment of colostrum quality.

5.2 INTRODUCTION

Colostrum is the initial secretion from the mammary gland after parturition, and is an important source of immunity and nutrition for newborn calves (Bielmann et al., 2010). Colostrum contains a high concentration of immunoglobulins (particularly IgG_1) that are absorbed by calves during the first 24 h of life (Baumrucker et al., 2010). Calves that fail to ingest and absorb a sufficient amount of colostral IgG are classified as suffering from failure of transfer of passive immunity (FTPI) (Godden, 2008). As a result of FTPI, a calf's susceptibility to infection, morbidity and mortality are increased (Robison et al., 1988; Donovan et al., 1998; Virtala et al., 1999). There are many factors associated with FTPI in newborn calves. The most important factors are related to colostrum management practices, including colostrum quality, volume, cleanliness and time of feeding after birth (Godden, 2008; Beam et al., 2009). Therefore, measuring IgG concentration in colostrum prior to feeding is a useful management tool that may be used to improve calf health. Monitoring also provided herd level information on colostrum quality that can lead to investigation of areas such as dry cow nutrition and transition cow management.

Several techniques have been developed to measure IgG concentration in colostrum, including laboratory based techniques such as the radial immunodiffusion (RID) assay (McBeath et al., 1971), high-performance liquid chromatography (HPLC) (Dolman and Thorpe, 2002) and electrophoresis (Page and Thorpe, 2002), and farm based techniques such as using a colostrometer (Bartier et al., 2015), refractometry (Bielmann et al., 2010) and colostrum color (Arguello et al., 2005). The RID assay is the most widely accepted reference method for quantification of colostral IgG (McBeath et

al., 1971). However, this assay has significant drawbacks, which are shared by most laboratory methods, including the time it takes to obtain the result (18 -24 h), lack of automation, utilization of reagents with a short shelf-life, high cost (Riley et al., 2007; Bielmann et al., 2010), and variability among kits due to inaccuracies associated with the internal standards (Ameri and Wilkerson, 2008). On-farm methods such as colostrometer and refractometry have been used to differentiate between good and poor quality colostrum (Bielmann et al., 2010; Bartier et al., 2015). Unfortunately, the colostrometer has several drawbacks as it is fragile, needs to be cleaned before each use and outputs are very sensitive to colostrum temperature (Bielmann et al., 2010; Bartier et al., 2015). These factors limit its use. For example, only 4% of farms in Quebec were reported to use the colostrometer consistently (Vasseur et al., 2010). Unlike the colostrometer, refractometry is not sensitive to temperature, but has shown a lower correlation with the reference RID method (Bielmann et al., 2010; Bartier et al., 2015). Therefore, an accurate, simple, rapid and cost-effective method to measure IgG content that overcomes the limitations of the reference and other methods is essential to assess colostrum quality.

The measurement of molecular concentrations in biological fluids by transmission infrared (IR) spectroscopy has a number of advantages that overcome the drawbacks associated with the other analytical methods. It is rapid (turnaround time is ~5 minutes), requires minimal sample preparation, and one spectrum can be used for quantitative analysis of several components (Shaw et al., 1998; Shaw and Mantsch, 1999; Shaw and Mantsch, 2000). For these reasons, transmission IR spectroscopy in combination with partial least squares (PLS) regression has been widely used for quantitative and qualitative analysis of biological specimens in human and veterinary medicine (Shaw and

Mantsch, 2000). For example, this approach provides the basis to simultaneously quantify total protein, glucose, albumin, triglyceride, cholesterol, and urea in human serum samples (Ward et al., 1989; Budinova et al., 1997; Shaw et al., 1998). In veterinary medicine, analytical methods based upon transmission IR spectroscopy have been proven suitable for milk analysis (Rutten et al., 2011a; Rutten et al., 2011b), determination of feed composition and the kinetics of nutrient degradation in the rumen (Herrero et al., 1997; Ohlsson et al., 2007), quantification of IgG concentrations in bovine (Elsohaby et al., 2014), equine (Riley et al., 2007) and alpaca sera (Burns et al., 2014), screening for metabolic diseases such as ketosis (Hansen, 1999) and for the detection of foodborne pathogens (Davis and Mauer, 2010). To the authors' knowledge, no study has used transmission IR spectroscopy in combination with PLS regression as the basis to quantify colostral IgG.

The objectives of this study were to develop a transmission IR-based analytical method for the quantification of bovine colostral IgG, and to demonstrate the utility of the new method for the assessment of poor quality colostrum (IgG <50 g/L).

5.3 MATERIALS AND METHODS

5.3.1 Colostrum samples

Postpartum milking colostrum samples were collected from 251 Holstein dairy cows originating from 7 commercial dairy herds in Prince Edward Island, Canada. Of these, 168 samples were collected between June and October 2013, while the remaining 83 samples were collected between May and August 2014. At each farm, the owner collected 50 mL of colostrum in a vial labeled with the identity of the cow and date of

sample collection, and then stored on the farm at -20°C until transportation to the Maritime Quality Milk Laboratory, University of Prince Edward Island (UPEI). There samples were stored at -80°C for later batch analysis. Animal care and ethics approval by the Animal Care Committee at UPEI was obtained for all procedures followed the Canadian Council on Animal Care (CCAC) guidelines (Care, Canadian Council On Animal, 2009).

5.3.2 RID assay for IgG

Colostrum samples were thawed at room temperature (20°C – 24°C) and vortexed for 10 s. These were then diluted (1:3) with deionized sterile water and mixed by vortexing at a maximum of 2,700 rpm for 10 s. After dilution, a commercial Bovine IgG RID Kit (Triple J Farms; Bellingham, WA) was used as the reference method for measuring colostral IgG concentration. The RID assay was performed according to manufacturer's instructions, using 5 μL of diluted colostrum in each well, tested alongside the manufacturer's reference standards. The same standards (same lot) were used on all RID assays. The RID plates were incubated at room temperature for 18 - 24 h, and the precipitating ring diameter surrounding the well was measured using a hand-held calliper. Each of the colostrum samples and assay standards was tested in replicates of five. The averages of the assay IgG standard concentrations was used to build a calibration curve from which the IgG concentration for each colostrum sample was determined. The final IgG concentration for each sample was determined by calculating the average of the 5 replicates. After the initial dilution, if samples had an IgG concentration greater than the manufacturer's stated performance range for the assay

(>3,000 mg/dL), the raw colostrum sample was diluted (1:5) with deionized sterile water and retested.

5.3.3 Transmission IR spectroscopy

Thawed colostrum samples were diluted (1:3) with deionized sterile water and mixed by vortexing at a maximum of 2,700 rpm for 10 s to ensure a uniform dispersion of colostral components. After dilution, each colostrum sample was tested in replicates of six by evenly spreading 5 μL aliquots of diluted colostrum onto 5 mm diameter wells within an adhesive-masked, 96-well silicon microplate (Riley et al., 2007). For each microplate, an empty well served as the background reference. The loaded microplates were allowed to dry at room temperature (20 - 24°C) for 2 h until the water had evaporated and a thin film formed, before being loaded into a multisampler (HTS-XT Autosampler, Bruker Optics) interfaced with a Bruker IR spectrometer (Tensor 37, Bruker Optics) equipped with a deuterium tryglycine sulfate detector and controlled by proprietary software (OPUS version 6.5, Bruker Optics). A total of 1,506 (251 samples x 6 replicates/sample) spectra were collected over the wavenumber range of 4,000 – 400 cm^{-1} with a nominal resolution of 4 cm^{-1}, co-adding 512 scans for each spectrum.

5.3.4 Spectra analysis

5.3.4.1 Spectral pre-processing

Absorbance spectra were converted into a printable format (PRN) (GRAMS/AI version 7.02, Thermo Fisher Scientific). The PRN format spectral data were imported into MATLAB version R2012b (MathWorks) and further data analysis was performed

using scripts written by the authors. First order derivative spectra were evaluated using the Savitzky–Golay method (smoothed with a second order polynomial function with nine point smoothing) (Savitzky and Golay, 1964) and then normalized using a vector normalization procedure (Barnes et al., 1989; Barnes et al., 2004) to reduce the effect of light scattering. This was followed by spectrum region selection, isolating the 3,700 – 2,600 cm^{-1} and 1,800 – 1,300 cm^{-1} wavenumber regions on the basis of their exhibiting the strongest absorptions in the original spectra. With 6 replicate spectra per colostrum sample, spectrum outlier detection was carried out using Dixon's Q-test (Dean and Dixon, 1951; Rorabacher, 1991; Rorabacher, 1991) at each wavenumber with a confidence level of 95%. Spectra with over 50% of the absorbance values detected as outliers were excluded from further analysis. The average spectrum for each sample was then used for the subsequent analysis.

5.3.4.2 Calibration model development

A multivariate regression method (PLS) was used to create a calibration model to relate the spectra obtained by transmission IR spectroscopy to the colostral IgG concentrations obtained by the reference RID method. Colostrum samples with IgG concentrations outside of the manufacture's stated performance range for the RID assay after dilution were excluded from further analysis (n = 1). The remaining 250 colostrum samples were used for building and validation of the analytical method. The colostral IgG concentrations obtained by the RID method were linked to their corresponding pre-processed IR spectra, and then randomly divided into test and calibration sets comprising one-third and two-thirds of the samples, respectively. The test set of spectra from

colostrum samples (n = 83) was identified by sorting all the colostrum samples according to their corresponding RID IgG concentration. The spectrum of every third colostrum sample was selected as a member of the test set, and the remainder (n=167) assigned to the calibration set. This approach was chosen to ensure that the test set encompassed the full range of IgG values in testing the predictive performance of the new analytical method. The calibration set (n = 167) was further split randomly into training (n = 84) and validation (n = 83) data sets for model development.

PLS regression was applied to the training set to develop 30 trial calibration models with the number of PLS factors ranging from 1 to 30. For each trial model, the sum of squares of the errors was calculated for the validation set. This procedure was repeated 10,000 times, with each repetition including new randomly assigned splits of the calibration data set into new training and validation sets. The root mean squared error for the Monte Carlo cross-validation value (RMMCCV) (Richard R. Picard and R. Dennis Cook, 1984; Xu and Liang, 2001) was calculated for each of the 30 trial calibration models using the following equation:

$$\text{RMMCCV} = \sqrt{\frac{1}{N n_{\text{v}}} \sum_{i=1}^{N} \left\| \mathbf{y}_i - \hat{\mathbf{y}}_i \right\|^2}$$

where N denotes the number of repeated procedures (N = 10,000), n_{v} is the number of samples in the validation set (n_{v} = 83), and $\mathbf{y}_i$ and $\hat{\mathbf{y}}_i$ represent the IgG concentrations for the validation set samples as obtained from RID experiments and predicted from the IR spectroscopic data, respectively. The optimal number of PLS

factors was chosen as the one giving the lowest RMMCCV value. Once the number of PLS factors had been determined, the training and validation sets were recombined to build the final calibration model.

5.3.4.3 Prediction of IgG concentration

The predictive performance of the new calibration model was assessed using the independent spectral data test set (n = 83). Scatter plots, Pearson correlation coefficients and concordance correlation coefficients (Lin, 1989) were calculated and used to assess the level of agreement between IgG concentrations obtained by the reference RID method and predicted by IR-based assay for both the calibration and test data sets. The difference and interchangeability between the RID and IR assays for the test set was further evaluated by the Bland-Altman plot (Altman and Bland, 1983; Bland and Altman, 1995; Bland and Altman, 1995).

The precision of both the RID and IR methods was investigated using the test data set. The mean and standard deviation (SD) were calculated from the replicate measurements for each colostrum sample, and from these a modified coefficient of variation (CV* = CV (1 + (1/4n)); n = 6 for IR assay and n = 5 for RID assay) was determined for each sample (Salkind, 2010), and for each analytical method. The CV* values were then plotted against the corresponding mean IgG concentrations.

The IR-based assay was further assessed using the ratio of predictive deviation (RPD: the RID-determined IgG concentration standard deviation, ratioed to the root mean squared error of prediction (RMSEP) for the IR-based assay), and the range error ratio (RER: the RID-determined IgG concentration range, ratioed to the RMSEP for the IR-

based assay) (Williams and Sobering, 1996). According to this framework, a RPD <2 is considered to be poorly predictive; values between 2.0 and 2.5 are adequate for qualitative evaluation or for screening purposes; values >2.5 (or RER >10) are regarded as acceptable for quantification; and values >3 (or RER >20) suggest that a model is suitable for very accurate quantitative analysis (Williams and Sobering, 1996).

5.3.5 Assessment of colostrum quality

To evaluate the potential applicability of the IR assay for assessing poor quality colostrum, we adopted RID-based colostral IgG <50 g/L (McGuirk and Collins, 2004) the cut-off value to differentiate between "poor" and "good" quality colostrum. The diagnostic sensitivity, specificity and accuracy were then evaluated for both the test set and the entire data set.

5.4 RESULTS

5.4.1 RID assay

The IgG content of the 250 colostrum samples (as determined by the RID assay) ranged from 4 to 145 g/L, with an average of 48 g/L and SD of 28 g/L. Only 39% of the colostrum samples had an IgG concentration above the standard cut-off point of 50 g/L for good quality colostrum.

5.4.2 IR spectra

Colostrum IR spectra showed characteristic absorption peaks over the wavenumber range of 4,000 to 400 cm^{-1} (Figure 5.1). A broad, strong absorption band centered at

3,300 cm^{-1} can be attributed to protein N-H stretching vibration (amide A), and other protein absorptions correspond to stretching vibrations of C=O (amide I) and bending vibrations of N-H (amide II) were centered around 1,650 and 1,550 cm^{-1}, respectively. Fat contributed very strong absorptions at 2850 and 2920 cm^{-1} (CH_2 stretching modes) and at ~1745 cm^{-1} (C=O stretch).

5.4.3 PLS calibration and validation

The PLS calibration model including 13 PLS factors was selected as optimal, based on the lowest RMMCCV (16 g/L). This model was based upon the first derivative spectra with nine points smoothing (Figure 5.2) and vector normalized to the 1,600 – 1,800 cm^{-1} region. The parameters and performance characteristics for the 13-factor model applied to both the calibration and test data sets are presented in Table 5.1. A scatter plot (Figure 5.3) represents the level of agreement between colostral IgG concentrations obtained by the RID assay and predicted by the IR-based assay. The Pearson (*r*) and the concordance correlation coefficients for both the calibration and test data sets were 0.91. The Bland-Altman plot (Figure 5.4) shows that the mean value of the difference between IgG concentrations obtained by IR and RID assays for the test data set was – 0.5 g/L, which is close to zero, indicating no significant systematic bias between the two methods. However, the 95% confidence interval ranged from -25 g/L to 24 g/L. Precision of the IR-based assay and RID method to measure the IgG content of the colostrum samples of the test data set are presented graphically in Figure 5.5A and Figure 5.5B, respectively. The mean CV* for the IR-based analyses was 8.9% and the mean CV* for RID analyses was 5.3%. The RPD and RER values were 2.3 and 11, respectively (Table 5.1).

5.4.4 IR assay sensitivity and specificity

For the test data set (n = 83), 51 (61.5%) colostrum samples had RID IgG concentrations below the threshold value of 50 g/L, and were therefore classified to be of poor quality. The sensitivity, specificity and accuracy of the IR-based assay were 88% (95% CI = 76 – 96), 94% (95% CI = 79 – 99) and 90%, respectively. Similar values were obtained when considering the entire data set (n = 250), 153 (61%) colostrum samples had an RID determined IgG content below 50 g/L; the sensitivity was 90% (95% CI = 84 – 94), specificity was 92% (95% CI = 84 – 96) and accuracy was 90%.

For the entire data set, the IR-based assay misclassified 24 samples; 8 were false positives and 16 were false negatives samples (Figure 5.3).

5.5 DISCUSSION

Colostrum is an important source of immunity and nutrition for newborn calves. Its quality (colostral IgG level) is one of the most important factors influencing calf health and future productivity (Bielmann et al., 2010). To address the need for a rapid, accurate analytical technique, this study aimed to develop a transmission IR spectroscopy-based method for the quantification of bovine colostral IgG concentration and thus to assess colostrum quality. To build a PLS calibration model with a high predictive ability, the data set should have a wide and evenly distributed composition (Murray, 1986). In this study, the IgG content of the colostrum samples was highly variable, ranged from 4 to 145 g/L, and more than half of the samples had IgG concentration <50 g/L (poor quality colostrum). Samples were farmer collected and while they were instructed to collect first

feeding samples it is possible that second or later feeding samples were included. However, the large range of IgG concentration values is beneficial for the current study of validating a new assay for assessing colostrum quality but may not reflect the industry norms for first fed colostrum.

The spectra of colostral samples showed complex absorption patterns and baseline shifts (Figure 5.1). This variability arises from the compositional complexity and variability of colostrum, which encompasses high and variable concentrations of various proteins, carbohydrates, lipids, and vitamins (Kehoe et al., 2007). Additional considerations include the high fat content of colostrum results in IR scattering (Norris, 2001), and residual hydration of the thin films. Spectral pre-processing was applied to the spectral data to decrease scatter, enhance bands, reduce band shifts (Figure 5.2) and improve the performance of the IR-based assay (Zeaiter et al., 2005).

The level of agreement between the IgG concentrations obtained by the RID method and predicted by the IR-based method was evaluated using a scatter plot, Pearson and concordance correlation coefficients and Bland-Altman plot. All methods confirmed good agreement between the two assays. In a recent study (Rivero et al., 2012) near-infrared spectroscopy was used to predict the IgG concentration of bovine colostrum and a slightly higher Pearson correlation coefficient (r = 0.95) was reported. This may be attributed to the lower number of colostrum (n = 157) samples and the smaller proportion (13%) of samples with IgG <50 g/L used for model development for that study. In contrast for the current study, 250 colostrum samples were used and almost five times as many samples had an IgG concentration <50 g/L. As a result, the PLS model development was weighted towards the colostrum samples with a low IgG content, which

is most important for the detection of poor quality colostrum (Weaver et al., 2000; Filteau et al., 2003; Godden, 2008).

The precision of the IR-based assay was somewhat lower than that of the reference RID assay. The relatively higher CV* (Figure 5.5A) of the IR method typically occurs because the samples in the test set were not involved in the calibration model development. However, the calibration model is considered very reliable if the coefficient of variation is lower than 10% (Albanell et al., 1999; Navrátilová et al., 2006; Navrátilová et al., 2006). In addition, the CV* of the IR-based assay lies within the acceptable range of the quality control standards of the US Food and Drug Administration Agency (US Department of Health and Human Services, 2001).

The utility of the calibration model for quantification of colostral IgG concentration from the test set samples was assessed using RPD and RER. With a RPD value of 2.3 and RER value of 11, this calibration model could be used to predict IgG content of bovine colostrum (Williams and Sobering, 1996; Williams, 2001; Williams, 2001). The RPD and RER values were lower than that reported in the other recent study which used near-infrared spectroscopy for the prediction of IgG content of bovine colostrum (Rivero et al., 2012).

Currently, there are several assays used to assess colostrum quality including the RID assay and on-farm tools such as the colostrometer and refractometry. The IR-based assay showed excellent correlation with the reference RID method, and better sensitivity and specificity than either the colostrometer or the refractometer (Bartier et al., 2015). The Pearson correlation between IgG concentration obtained by RID and IR methods (r = 0.91) was higher than that reported for colostrometer (r = 0.77) and Brix refractometer (r

= 0.64) (Bartier et al., 2015). The sensitivity (90%) and specificity (92%) of the IR method were higher than the sensitivity (84% and 66%) and specificity (77% and 83%) recently reported for the colostrometer and Brix refractometer, respectively (Bartier et al., 2015). The great majority of colostrum samples were correctly classified by the IR-based assay (Figure 5.3) using RID IgG 50 g/L (McGuirk and Collins, 2004) as the cut-off value to differentiate between poor and good quality colostrum. The IR-based assay misclassified 9.6% of 250 samples, of most concern was the 16 false negatives (6.4%). These false negative samples have RID IgG concentration ranged between 35 – 49 g/L, which is relatively close to the cut-off value of 50 g/L (Figure 5.3) and thus a low risk of FTPI would be expected if sufficient volume of these colostrum meals were fed to newborn calves (McGuirk and Collins, 2004).

5.6 CONCLUSIONS

The assessment of colostral IgG concentration serves a useful management tool that may be used to improve colostrum management practices and calf health. Methods for accurate, rapid and cost-effective measurement of colostral IgG are needed. The PLS calibration model in combination with transmission IR spectroscopy shows potential as an accurate, rapid, reagent-free and inexpensive laboratory-based method for quantification of bovine colostral IgG concentration, and thus for assessment of bovine colostrum quality.

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Table 5.1. Descriptive statistics for the colostral immunoglobulin G concentrations obtained by the reference radial immunodiffusion (RID) assay and for the best partial least squares (PLS) transmission infrared spectroscopy calibration model

Data sets	*n*	RID-determined IgG[a]			PLS model characteristics[b]				
		Mean	SD	Range	PLS factors	*r*	RMSEC/ RMSEP	RPD	RER
Calibration	167	48.0	28.4	140.4	13	0.91	12.3	-	-
Test	83	47.4	27.6	134.2	13	0.91	12.2	2.3	11

[a] Mean = average of RID data (g/L); SD = standard deviation of RID data (g/L); range = difference (highest minus lowest) of RID data (g/L)

[b] *r* = Pearson correlation coefficient; RMSEC = root mean squared error of calibration (g/L); RMSEP = root mean squared error of prediction (g/L); RPD (ratio of predictive deviation) = SD divided by RMSEP; RER (range error ratio) = range divided by RMSEP.

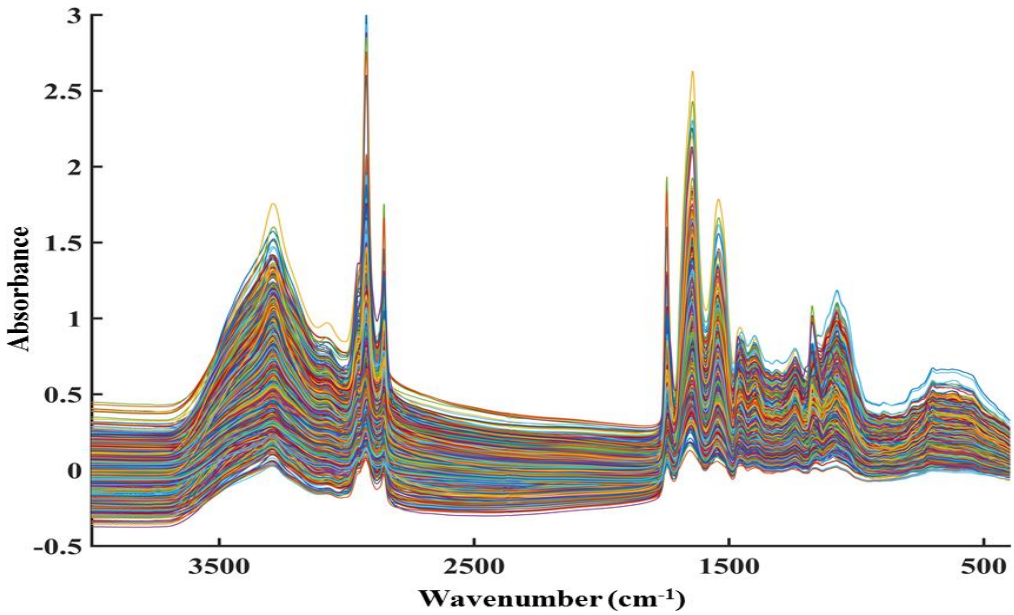

Figure 5.1. Raw infrared spectra of 250 colostrum samples over the spectral range of 400 to 4000 cm^{-1}. The strong protein absorptions cantered around 3300, 1650 and 1550 cm^{-1} were attributed to N-H stretching (Amide A), C=O stretching (Amide I) and N-H bending (Amide II), respectively.

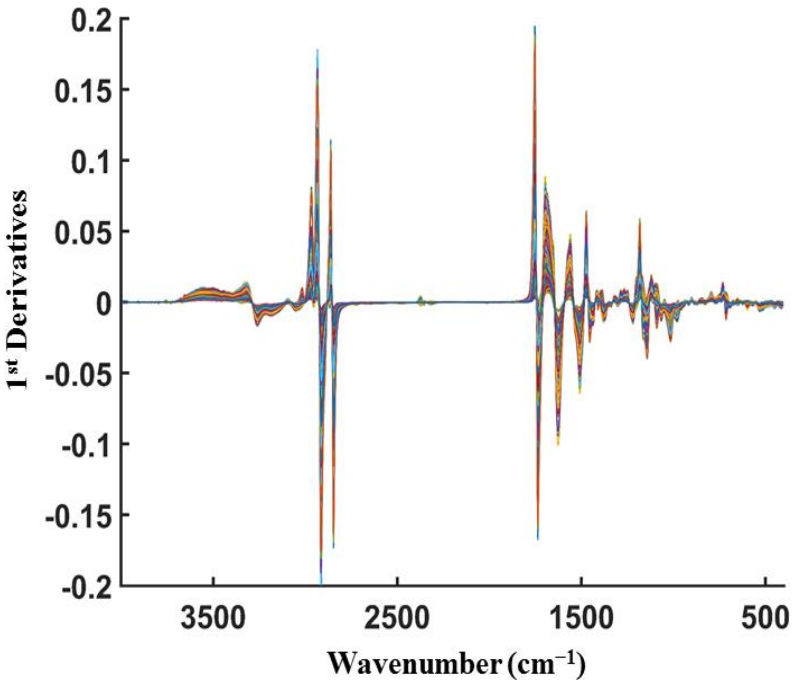

Figure 5.2. First derivatives of 250 bovine colostrum spectra with nine points Savitzky-Golay smoothing.

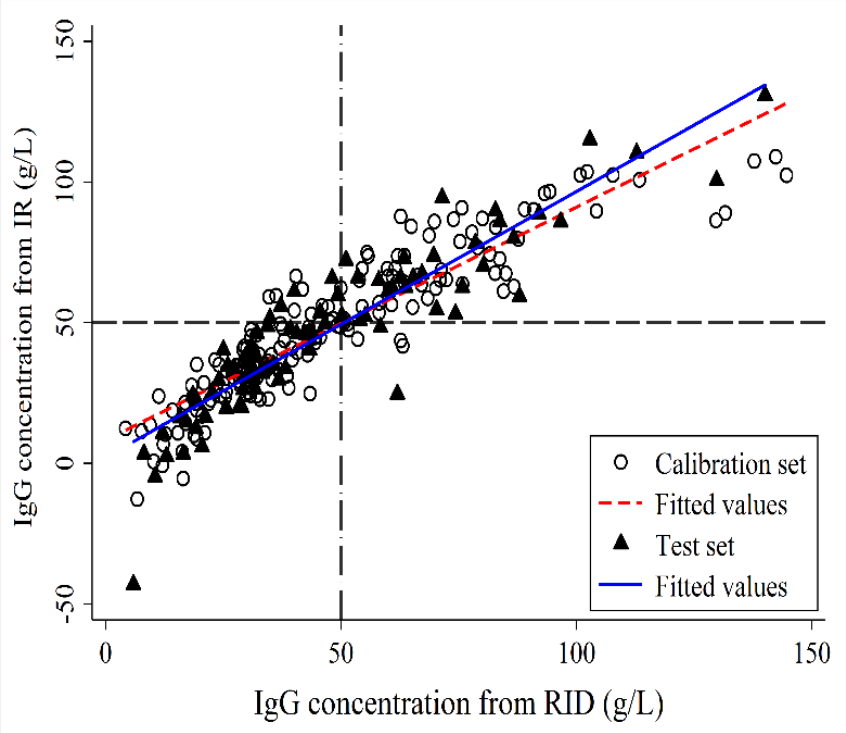

Figure 5.3. Scatter plot comparing colostral IgG concentrations obtained by the IR-based assay and radial immunodiffusion (RID) assay for the calibration and test data sets. The lines represents the best fit for calibration (dashed line) and test set (solid line). The long dashed lines represent the cut-off value (50 g/L) between poor and good quality colostrum.

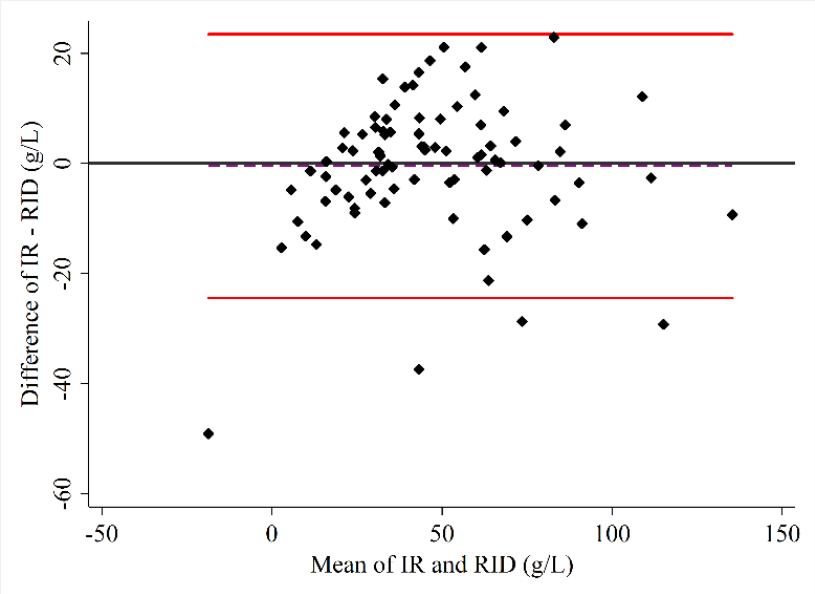

Figure 5.4. Bland-Altman plot of the difference in the colostral IgG concentrations obtained by IR-based assay and radial immunodiffusion (RID) assay for the test set (n = 83). The solid lines represent the 95% confidence limits of agreement (–25 to 24 g/L). The horizontal dashed line represents the mean difference between IR and RID assays (-0.5 g/L).

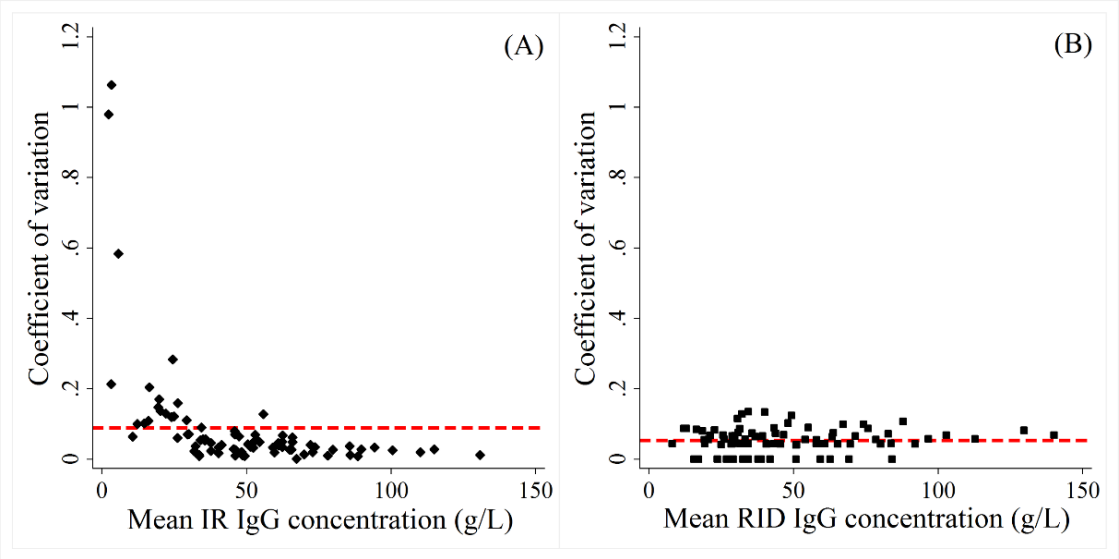

Figure 5.5. Modified coefficient of variance (CV*) plots for (A) the IR-based assay; (B) the radial immunodiffusion (RID) assay. The dashed lines represent the mean CV* for the IR-based assay (8.9%) and the RID assay (5.3%).

CHAPTER 6

PRELIMINARY VALIDATION OF A CALF-SIDE TEST FOR DIAGNOSIS OF FAILURE OF TRANSFER OF PASSIVE IMMUNITY IN DAIRY CALVES

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6.1 ABSTRACT

The objective of this study was to evaluate the utility of an initial version of a calf-side test (ZAPvet Bovine IgG test) for diagnosis of failure of transfer of passive immunity (FTPI) in dairy calves. Blood samples (n = 202) were collected from calves from 1 to 11 d of age. Serum IgG concentration was determined by radial immunodiffusion (RID) assay. The mean IgG concentration was 1,764 + 1,035 mg/dL, with a range from 133 to 5,995 mg/dL. The ZAPvet Bovine IgG test was used to assess FTPI (serum IgG < 1,000 mg/dL) and test characteristics were calculated. The number of samples that had FTPI from the RID assay and ZAPvet test was 55 and 96 samples, resulting in a true prevalence of 27% and an apparent prevalence of 47.5%, respectively. The sensitivity, specificity, positive and negative predictive values of the ZAPvet test were 0.82, 0.65, 0.47 and 0.91, respectively. The results of the ZAPvet test were derived from two observers, and the overall level of agreement between the results of the two observers was 84%, with a kappa-value of 0.67. The ZAPvet Bovine IgG test showed good potential for further development as a calf-side, rapid, and cost-effective test for monitoring FTPI in dairy calves.

6.2 INTRODUCTION

Newborn calves are born agammaglobulinemic, without circulating immunoglobulin G, and are dependent on the passive transfer of IgG from maternal colostrum provided within the first hours of life (Smith et al., 1964). Calves which fail to ingest and/or absorb sufficient colostral IgG, resulting in a serum IgG concentration < 1,000 mg/dL, suffer from a condition named failure of transfer of passive immunity (FTPI) (Vogels et al., 2013).

FTPI in dairy calves is a significant problem (Fecteau et al., 2013), and is considered a predisposing risk factor for most early neonatal infectious disease losses and long-term productivity losses in heifers. Additionally, the impact of FTPI can be observed as decreased milk yield and increased culling rates in first lactating animals (Tyler et al., 1996b; Godden, 2008). The reported prevalence of FTPI in dairy calves in North America ranged from 19% to 40% (Nocek et al., 1984; Dairy, 2007). Having calves with lower levels of passive immunity has major economic consequences for dairy producers (Trotz-Williams et al., 2008) including reduced calf daily gains (Robison et al., 1988), increased morbidity and mortality, increased production costs and reduced profitability for the dairy industry (Morin et al., 1997; Jaster, 2005). Therefore, monitoring of FTPI rates can identify herd management deficiencies and ensure timely detection and medical interventions, which are important to reduce the risk of associated diseases among calves and economic impact for dairy producers (Furman-Fratczak et al., 2011).

Several assays are available to assess passive immunoglobulin transfer status in newborn calves (Tyler et al., 1996a; Parish et al., 1997; Weaver et al., 2000). The radial immunodiffusion (RID) assay is the reference standard method for determining passive

immunoglobulin transfer by measuring the quantity of IgG in the serum (Weaver et al., 2000; Godden, 2008). The RID assay is time-consuming and cannot be performed by producers or veterinarians on-farm, as it is a laboratory procedure that requires trained laboratory technicians. The procedure takes 18 - 24 h to obtain results, requires advanced technical skills and care to perform the assay and to measure zones of precipitation accurately and is not amenable to automation (Liu et al., 2007). Additionally, RID utilizes reagents with a limited shelf life, and is often more expensive than the indirect assay methods (Riley et al., 2007; Bielmann et al., 2010). Imprecision in the RID assay replicates from the same sample have been noted and are attributed to inconsistencies in the assay standards (Ameri and Wilkerson, 2008). Other than RID, serum total protein determination using refractometry, sodium sulfite turbidity test, zinc sulfate turbidity test, ELISA, serum γ-glutamyl transferase activity, and whole blood glutaraldehyde coagulation test have been described with varying degrees of accuracy for predicting IgG concentrations in calves (Tyler et al., 1996a; Parish et al., 1997; Weaver et al., 2000). Turbidimeteric immunoassay described by Etzel et al. (1997) performed well in calves (Dawes et al., 2002; Alley et al., 2012) and foals (Davis et al., 2005). However, these assays require specific instruments and specially trained personnel to perform data interpretation.

Therefore, methods for accurate, rapid, convenient, and cost-effective calf-side diagnosis and simple monitoring of FTPI in calves are needed. The ZAPvet Bovine IgG Test (ZBx Corporation, Toronto, Canada) is intended to be a fast and simple on-farm test to confirm adequate passive immunity transfer in calves. The ZAPvet Bovine IgG Test uses two visual lines, a test line and a reference line, to provide a semi-quantitative

determination of the concentration of bovine IgG using whole blood, plasma, or serum samples. The ZAPvet Bovine IgG Test was developed using calibrators with turbidimeteric determined concentrations. The ZAPvet test is inexpensive and rapid. It does not require any instrument or reagent/buffer, and therefore, can be performed anywhere and at anytime. The objective of this study was to describe the test characteristics of the initial version of a ZAPvet Bovine IgG test against RID, and measure inter-observer variations for assessing FTPI in dairy calves when using serum.

6.3 MATERIALS AND METHODS

6.3.1 Serum samples

Holstein calves (n = 202) from 5 commercial dairy herds in Prince Edward Island and one herd in Nova Scotia were sampled between June and October 2013. Whole blood was collected from 1 to 11 d old calves by jugular venipuncture, using a 20-gauge, 1-inch hypodermic needle (BD Vacutainer Precision Glide, Becton Dickinson Co., Franklin Lakes, NJ), into a sterile, plastic Vacutainer tube without anticoagulant (BD Vacutainer, Becton Dickinson and Co.). Samples were transported on ice in a cooler to the Maritime Quality Milk Laboratory, University of Prince Edward Island (UPEI). Serum was separated by centrifugation at 1,500 g for 10 min at ~20 °C within 5 h of collection. Three aliquots of serum were collected and stored at -80 °C. Serum from Nova Scotia samples was separated by similar centrifugation methods at a local veterinary clinic and then frozen at -20 °C until transport to UPEI. This study was conducted in accordance with the Canadian Council on Animal Care (CCAC) guidelines (Care, Canadian Council

On Animal, 2009) under a protocol (No. 6005332) approved by the Animal Care Committee at UPEI.

6.3.2 Radial immunodiffusion assay (Reference standard)

Serum samples were allowed to thaw at room temperature (20 – 24 °C) and vortexed for 10 s. Subsequently, IgG was measured by RID (Bovine IgG RID Kit, Triple J Farms, Bellingham, WA). The RID assay was performed according to manufacturer's instructions, using 5µL of undiluted serum sample in each well. The same lot number plates were used on all RID assays to reduce variability (SD) in the assay results. The diameter of precipitated rings was measured after 18 – 24 h of incubation at room temperature using a handheld caliper. Each of the samples and assay standards were tested in replicates of five. The averages of the five replicates of the assay standards were used to build a calibration curve that was subsequently used to determine IgG concentrations for the serum samples. The final IgG concentration for each sample was determined by calculating the average of the five replicates. Serum samples with IgG concentrations greater than the manufacturer's stated performance range for the assay (> 3,000 mg/dL) were diluted (50:50) with deionized sterile water and retested.

6.3.3 ZAPvet Bovine IgG test

Serum samples were thawed at room temperature and vortexed for 10 s before being tested by ZAPvet Bovine IgG test (ZBx Corporation, Toronto, Canada). The ZAPvet test uses two visual lines, a test line and a reference line, to provide a semi-quantitative determination of the concentration of bovine IgG in serum samples (Figure

6.1). The ZAPvet test was performed according to manufacturer's instructions. Undiluted serum (35 – 40 µL) was added to the sample application zone. The test was kept on a flat surface and incubated at room temperature for 15 to 20 min, then read by comparing the intensity of the test line to the reference line (Table 6.1). The result was interpreted by two observers. Observer #1 (student) did the initial test reading and took a digital photo at the time of interpretation. The photo was sent to Observer #2 (ZBx scientist) for interpretation.

6.3.4 Statistical analysis

Statistical analysis was performed using Stata software (Stata Corporation, 2013), with results considered significant at $P < 0.05$. The analysis was performed in 3 stages. First, the test characteristics of the ZAPvet Bovine IgG test for diagnosis of FTPI in dairy calves were calculated using 2x2 tables (diagt command in Stata). Sensitivity (Se) was defined as the proportion of calves with FTPI, as determined by RID, that were classified as positive on ZAPvet test. Conversely, specificity (Sp), was defined as the proportion of calves without FTPI that were classified as negative on ZAPvet test. Accuracy was defined as the proportion of calves that were correctly classified by the ZAPvet test. The predictive values of a test are dependent upon the Se and Sp of the test and the prevalence of disease in the study population (Dohoo et al., 2009). In order to examine the performance of the ZAPvet test in different populations, with varying degrees of FTPI, the positive (PPV) and negative (NPV) predictive values were calculated for prevalence estimates ranging from 1 to 100%. The PPV was the proportion of ZAPvet test-positive calves that truly had FTPI, and the NPV was the proportion of ZAPvet test-negative

calves that did not have FTPI. Second, the test characteristics of the ZAPvet Bovine IgG test were assessed at different test line intensities (weaker, similar, and stronger), based on results derived from Observer #1 (Table 6.1), followed by calculation of percent of agreement with the RID assay. Finally, the level of agreement between results of the ZAPvet test derived from the two observers were assessed using McNemar's test (Lachenbruch and Lynch, 1998) for paired data to check for bias, followed by calculation of the kappa statistic.

6.4 RESULTS

6.4.1 RID assay

The mean and standard deviation for IgG concentrations of the serum samples (n = 202) as obtained by RID were 1,764 $\pm$ 1,035 mg/dL (ranging from 133 to 5,995 mg/dL). The number of samples with IgG concentrations below a cut-off value of 1,000 mg/dL for FTPI positive cases was 55 out of 202 samples (Figure 6.2), which generated a true FTPI prevalence of 27%.

6.4.2 ZAPvet Bovine IgG test

6.4.2.1 Agreement with RID

The number of samples with test line intensity weaker than the reference line (IgG < 1,000 mg/dL) was 96 out of 202 samples for Observer #1, resulting in an apparent FTPI prevalence of 47.5%. The ZAPvet test results derived from Observer #1, correctly identified 45 (82%) calves as FTPI positive and misclassified 10 (18%) calves as FTPI

negative that had IgG concentrations of less than 1,000 mg/dL on RID (false negative (FN)). A false positive (FP) result occurred in 51 (35%) calves that were FTPI negative and had IgG concentrations greater than 1,000 mg/dL, according to RID (Table 6.2). The mean and standard deviation for IgG concentration of FN and FP samples on RID were 866 $\pm$ 139 mg/dL, with range of 568 – 996 mg/dL and 1,746 $\pm$ 579 mg/dL, with range of 1,014 – 3,415 mg/dL, respectively (Figure 6.3).

Observer #2 identified 83 out 202 calves as having FTPI, resulting in an apparent FTPI prevalence of 41%. ZAPvet test results interpreted by Observer #2 correctly classified 40 (73%) of the FTPI positive calves and misclassified 15 (27%) calves as negative (FN). These 15 calves had a mean IgG concentration of 832 $\pm$ 166 mg/dL on RID with a range of 568 – 996 mg/dL. FP results occurred in 43 (29%) calves, which had a mean IgG concentration of 1,730 $\pm$ 564 mg/dL on RID with a range of 1,014 – 3,415 mg/dL.

6.4.2.2 Test characteristics and predictive values

Table 6.3 presents the test characteristics of the ZAPvet Bovine IgG test, including sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV). Accuracy for the ZAPvet test was determined for the assessment of FTPI (IgG < 1,000 mg/dL) based on the results derived from the two observers. The PPV was relatively low, while NPV was higher for both observers. Figure 6.4 shows the predictive ability of the ZAPvet test at various prevalences of FTPI, ranging from 1 to 100%.

6.4.2.3 Performance at different test line intensities

Using the observations from Observer 1, the number of samples with test line intensity weaker (IgG < 1,000 mg/dL), similar (between 1,000 – 2,000 mg/dL), and stronger (> 2,000 mg/dL) than the reference line, were 96 (47.5%), 98 (48.5%) and 8 (4%), respectively. The test characteristics of the ZAPvet test at weaker, similar, and stronger test intensities are presented in Table 6.4.

6.4.2.4 Inter-observer variations

Table 6.5 presents the FTPI determination inter-observer agreement of two observers using the ZAPvet test. The overall percent of agreement was 84%, with a corresponding kappa-value of 0.67. The percent of positive agreement was 69%. The McNemar's test for the sensitivity comparison between the two observers showed no significant difference ($P = 0.18$) between proportions of calves classified as having FTPI by the two observers. McNemar's test for the specificity comparison between the two observers also showed no significant difference ($P = 0.15$) between proportions of calves classified as having adequate transfer of passive immunity (ATPI).

6.5 DISCUSSION

Failure of transfer of passive immunity in dairy calves is associated with high morbidity and mortality. Monitoring of FTPI rates can identify herd management deficiencies, which are important to reduce the risk of associated diseases among calves (Furman-Fratczak et al., 2011). Therefore, a calf-side method with high Se is essential to correctly identify calves with FTPI for action such as additional monitoring and

intervention. The ZAPvet test had reasonably high Se (82%) and, as a result, dairy producers and veterinarians will be able to confirm ATPI to their calves on farms or in private veterinary clinics. Moreover, the ZAPvet test will give an indication of the prevalence of FTPI in the herd and will identify deficiencies in the colostrum management practices. Estimates of within-farm prevalence can be used to motivate producers to take action with respect to colostrum management and to monitor the effectiveness of changes in management. The Se and Sp of the ZAPvet test are very close to the Se (87%) and Sp (69%) detected for the same samples using optical refractometry in our laboratory with the same study population (data not shown) and similar to previous reports on refractometry (Deelen et al., 2014). The Se and Sp of the ZAPvet test was somewhat lower than that calculated for automated turbidimeteric immunoassay; however this test is not currently available for cow-side use (Etzel et al., 1997; Dawes et al., 2002; Alley et al., 2012).

Using ZAPvet test to assess FTPI in dairy calves has some advantages compared with refractometry. Once the serum sample is obtained, refractometry can diagnose FTPI in less than 15 s, but very few dairy farms have access to a centrifuge to separate serum. In this study, the ZAPvet test assessed FTPI using serum, but whole blood and plasma can also be used. This means that producers could take a blood sample and apply the ZAPvet test directly to ensure ATPI in their calves on-farm. Validation of the ZAPvet test for diagnosis of FTPI using whole blood is warranted. There is no initial investment for producers to begin monitoring FTPI with the ZAPvet test. Consumable costs for the test are estimated at $5-8 CDN, depending on the marketing channel. In comparison, the

upfront cost to begin monitoring with a good quality refractometer is approximately $350 CDN.

In the test population, the ZAPvet test correctly diagnosed the majority of FTPI cases, with a relatively low number of FN (10 samples). The FN samples had IgG concentrations measured by RID between 568 to 996 mg/dL, that were close to 1,000 mg/dL (Figure 6.3), indicating only partial FTPI, perhaps posing a lower risk of morbidity and mortality (Lee et al., 2008; Fecteau et al., 2013). The Sp (65%) of the ZAPvet test was moderate, with a relatively high number of FP (51 samples). Sixteen out of 51 FP samples had IgG concentrations measured by RID between 1,014 – 1,260 mg/dL (Figure 6.3), which is very close to 1,000 mg/dL. There are several possible reasons leading to disagreement between ZAPvet and RID results. It is well known that immunoassays are affected by many factors. First, different antibodies were used by these two assays. RID standards are made using adult bovine serum (IgG_1 and IgG_2), while ZAPvet test was made using calf serum (mainly IgG_1) (Butler, 1983). Changing the antibodies could greatly affect the result (Tate and Ward, 2004). Secondly, RID itself has substantial variability. The literature has shown that RID assays provided by different manufactures could give results that differ by as much as 40%. This is thought to be due to the differences in standards used to determine calibration curves (Ameri and Wilkerson, 2008). Considering such a high degree of variation between RID assays provided by different manufacturers, the difference between ZAPvet and RID is acceptable. In this study, each sample was tested in replicates of five by RID method to minimize variations and only one replicate by ZAPvet test. The mean coefficient of variation for the five replicates tested by RID method was 7.8% (data not shown).

Specificity of the ZAPvet test might be improved with recalibration of the device, but this is likely to come at the cost of lower Se. The relative importance of FP versus FN diagnoses and the predictive values in relevant populations should be considered prior to recalibration. In North America, the reported prevalence of FTPI in dairy calves ranged from 19% to 40% (Trotz-Williams et al., 2008; Dairy, 2007). Figure 6.4 illustrates the predictive values of a positive or negative ZAPvet test across populations with varying prevalences of FTPI. When the proportion of calves with FTPI increased, NPV decreased. For example, at a low prevalence of 19% and a high prevalence of 40%, the NPV of the ZAPvet test was 94% and 84%, respectively. Translating the NPV to the probability that a ZAPvet test negative calf truly has FTPI (positive predictive value of a negative test (PPVN = 1 − NPV; Dohoo et al., 2009)). Subsequently, the proportion of calves with FTPI that would not receive treatment will be very small (6%) for low prevalence herds and relatively large (16%) for high prevalence herds.

The PPV of the ZAPvet test was relatively low, and as a result a substantial number of calves with ATPI will be classified as having FTPI. Compared to the overall cost of the disease, which is estimated at $1105 CDN per affected calf (Stilwell and Carvalho, 2011), the cost of a false positive FTPI diagnosis is relatively low at the calf-level, very young calves (prior to complete gut closure) might receive additional colostrum or colostral supplements, as well as more intense monitoring for early intervention if disease occurs. At the herd-level, while prevalence may be overestimated, the relative impact of changes in management should be apparent, even if the predictive ability for individual animals is less than ideal.

The level of agreement between results derived from Observer #1 (Student) and Observer #2 (ZBx scientist) was high (84%), with a kappa value of 0.67, which is considered substantial agreement. This indicates that the two operators agree on the classification of calves with respect to calves with FTPI and ATPI (Table 6.5). The test characteristics of the ZAPvet test based on Observer #1 and Observer #2 derived results were very close (Figure 6.3 and 6.4), in agreement with the McNemar's test which showed non-significant difference ($P > 0.05$) between proportions of calves classified as having FTPI and ATPI by the two observers. The process of interpretation of the test line intensity in comparison with the reference line was made easy by the provision by the manufacturer of example photos for finished ZAPvet tests for samples with different IgG concentrations, ranging from 115 to 3,200 mg/dL. Consequently, producers will be able to perform the ZAPvet test with minimal training.

6.6 CONCLUSIONS

Preliminary data suggest that the ZAPvet Bovine IgG test has potential for monitoring FTPI on farms or in veterinary clinics. The ZAPvet test is relatively sensitive and would be acceptable as an initial screening test for diagnosis of FTPI in dairy calves, and is more efficient than submitting serum samples to an accredited veterinary diagnostic laboratory for IgG testing. However, the low specificity of ZAPvet test would result in over prediction of FTPI incidence, which could result in unnecessary interventions for calves with ATPI. Furthermore, studies to validate ZAPvet test for diagnosis of FTPI using whole blood samples are warranted.

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Table 6.1. Interpretation of test line intensity of the ZAPvet Bovine IgG test (ZBx Corporation, Toronto, Canada) corresponding to IgG concentrations measured by the radial immunodiffusion assay (Triple J Farms, Bellingham, WA).

ZAPvet test	RID test	Failure of transfer of passive immunity (FTPI)
Test line intensity	IgG conc. (mg/dL)	
Weaker	Less than 1,000	Positive case
Similar	Between 1,000 to 2,000	Negative case
Stronger	More than 2,000	Negative case

Table 6.2. Agreement between radial immunodiffusion assay (Triple J Farms, Bellingham, WA) and the ZAPvet bovine IgG test (ZBx Corporation, Toronto, Canada) for diagnosis of failure of transfer of passive immunity (IgG < 1,000 mg/dL) in 202 dairy calves based on results derived from two observers. Cells displaying the disagreement between two tests, resulting in the misclassification of calves with FTPI, are shaded.

Tests		ZAPvet test[1]				
		Observer #1			Observer #2	
		T+	T-	Total	T+	T-
RID test	T+	45	10	55	40	15
	T-	51	96	147	43	104
	Total	96	106	202	83	119

[1]Observer #1 = student; Observer #2 = ZBx scientist; T+ = test positives; T- = test negatives

Table 6.3. Test characteristics and predictive values of the ZAPvet Bovine IgG test (ZBx Corporation, Toronto, Canada) for assessment of FTPI (IgG < 1,000 mg/dL) in 202 dairy calves based on results derived from two observers.

Test Characteristics[1]	ZAPvet test	
	Observer #1	Observer #2
Pr	0.475	0.41
Se (95% CI)	0.82 (0.69 - 0.91)	0.73 (0.59 - 0.84)
Sp (95% CI)	0.65 (0.57 – 0.73)	0.71 (0.63 – 0.78)
PPV (95% CI)	0.47 (0.37 – 0.57)	0.48 (0.37 – 0.59)
NPV (95% CI)	0.91 (0.83 – 0.95)	0.87 (0.80 – 0.93)
Accuracy	0.70	0.71

[1]Pr = prevalence; Se = sensitivity; Sp = specificity; PPV = positive predictive value; NPV = negative predictive value; Accuracy = number of correctly classified calves.

Table 6.4. Test characteristics and predictive values of the ZAPvet Bovine IgG test (ZBx Corporation, Toronto, Canada) at different test line intensity levels.

Test line intensity	Test characteristics[1]			
	Se	Sp	PPV	NPV
Weaker	0.82 (45/55)	0.65 (96/147)	0.47 (45/96)	0.91 (96/106)
Similar	0.52 (39/75)	0.54 (68/127)	0.40 (39/98)	0.65 (68/104)
Stronger	0.10 (7/72)	0.99 (129/130)	0.88 (7/8)	0.67 (129/194)

[1]Se = sensitivity; Sp = specificity; PPV = positive predictive value; NPV = negative predictive value.

Table 6.5. Inter-observer agreement for ZAPvet Bovine IgG test (ZBx Corporation, Toronto, Canada) results derived from two observers for assessment failure of transfer of passive immunity in 202 dairy calves.

Observers		Observer #2		Total
		T+	T-	
Observer #1	T+	73	23	96
	T-	10	96	106
	Total	83	119	202

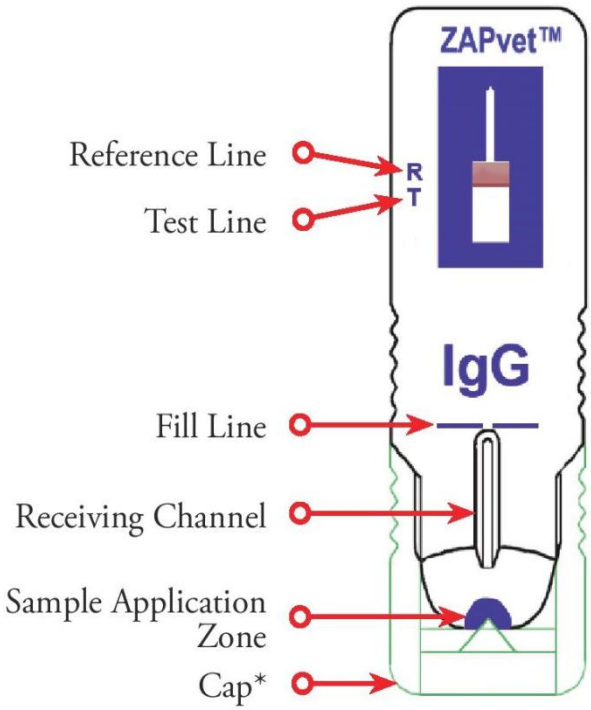

Figure 6.1. Illustration of the ZAPvet Bovine IgG test (ZBx Corporation, Toronto, Canada).

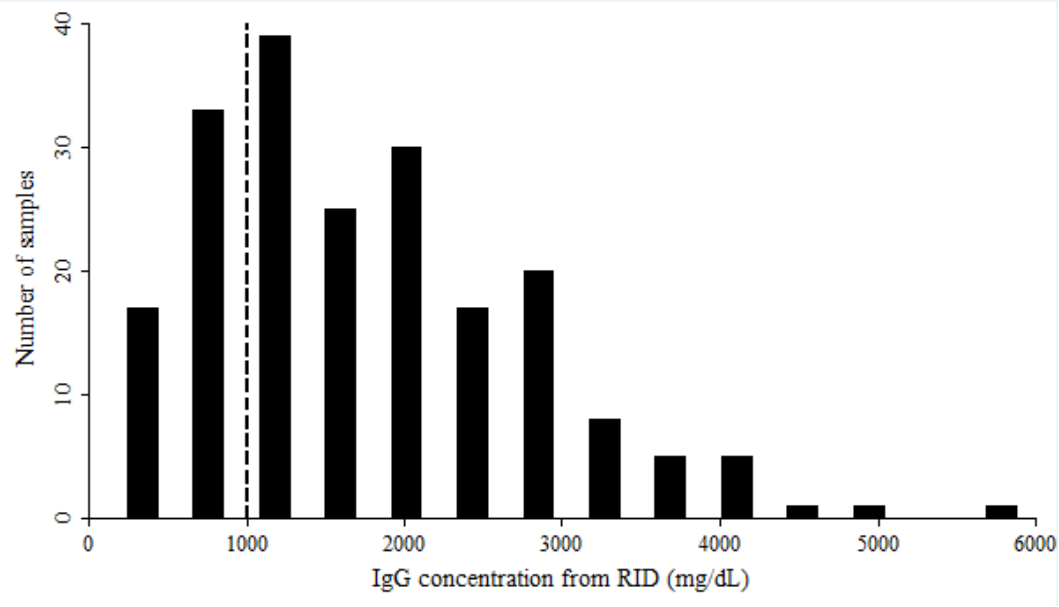

Figure 6.2. Histogram of the distribution of serum IgG concentrations for 202 calves, measured by the radial immunodiffusion assay (Triple J Farms, Bellingham, WA). The dashed line represents the cut-off value for failure of transfer of passive immunity positive cases (IgG < 1,000 mg/dL).

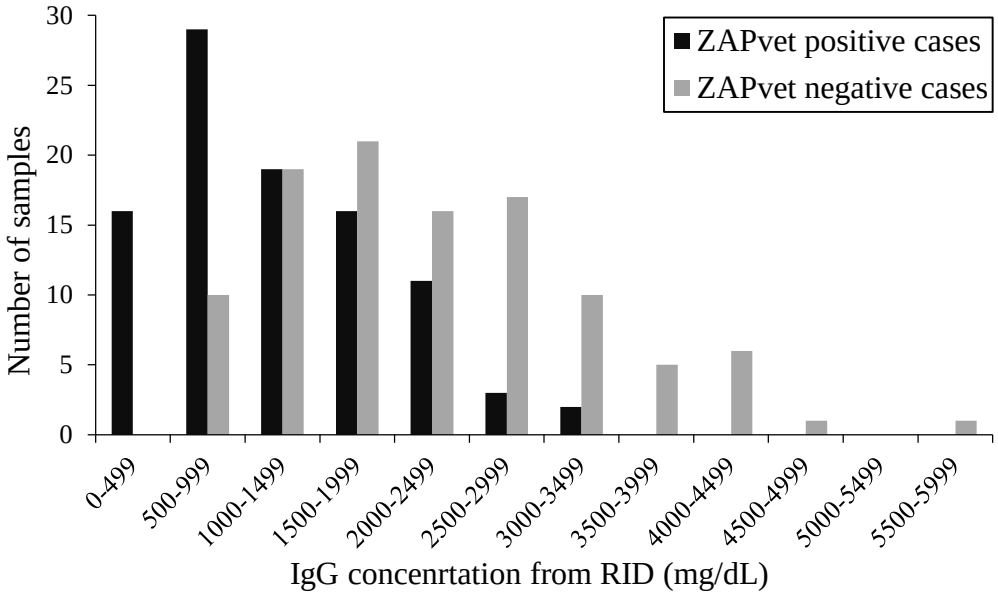

Figure 6.3. Histogram of the distribution of the ZAPvet Bovine IgG test (ZBx Corporation, Toronto, Canada) results derived from Observer #1 for diagnosis of failure of transfer of passive immunity in 202 calves, against serum IgG concentrations measured by radial immunodiffusion assay (Triple J Farms, Bellingham, WA). The dashed line represents the cut-off value for FTPI positive cases (IgG < 1,000 mg/dL).

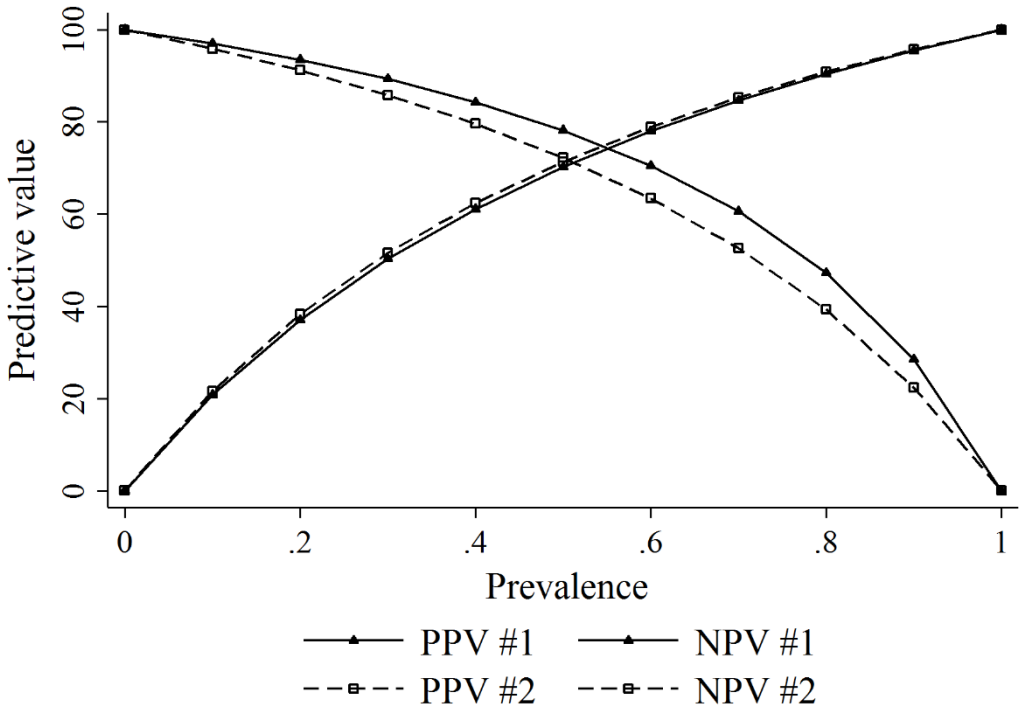

Figure 6.4. Positive predictive values (PPV) and negative predictive values (NPV) for the ZAPvet Bovine IgG test (ZBx Corporation, Toronto, Canada) based on results derived from two observers for diagnosis of failure of transfer of passive immunity (IgG < 1,000 mg/dL) in 202 dairy calves.

CHAPTER 7

EVALUATION OF DIGITAL BRIX AND OPTICAL STP REFRACTOMETERS FOR ASSESSING FAILURE OF TRANSFER OF PASSIVE IMMUNITY IN DAIRY CALVES

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7.1 ABSTRACT

This study evaluated the performance of and differences between a digital Brix refractometer and an optical STP refractometer for assessing failure of transfer of passive immunity (FTPI) in dairy calves. Blood samples (n = 200) were collected from Holstein calves from 1 to 11 days of age. Serum IgG concentration was measured by radial immunodiffusion (RID) assay. The percentages of brix (%Brix) and serum total protein (STP) were determined by digital Brix refractometer and optical refractometer, respectively. The mean of RID IgG concentrations was 1,765 mg/dL (SD ± 1,039), with a range from 133 to 5,995 mg/dL. The mean of %Brix concentrations was 8.8%Brix (SD ± 1.03), with a range from 5.9 to 12.9%Brix. The mean of STP concentrations was 5.98 g/dL (SD ± 1), with a range from 4.2 to 10.6 g/dL.

The RID IgG concentration was positively correlated with Brix percentage (r = 0.79) and with STP (r = 0.74). The correlation between Brix percentage and STP was (r = 0.73). Test characteristics of digital Brix and optical refractometers for assessment of FTPI (serum IgG < 1,000 mg/dL) using previously recommended cut-off values were calculated. The Se, Sp and accuracy of digital Brix refractometer, at a cut-point of 8.2%Brix, were 76.4, 85.5 and 83%, respectively. Similarly, the Se, Sp and accuracy of optical STP refractometer, at a cut-point of 5.2 g/dL, were 69.1, 89 and 83.5%, respectively. A receiver operating curve was created, the area under the curve (AUC) of digital Brix and optical STP refractometers were 0.89 and 0.88 respectively. The best combination of Se (85.5%), Sp (82.8%) and accuracy (83.5%) of digital Brix

refractometer was at 8.3%Brix. While, for optical STP refractometer the best combination of Se (80%), Sp (80.7%) and accuracy (80.5%) was at 5.5 g/dL.

The overall percent of agreement between results of digital Brix and optical STP refractometers was 85%, with a corresponding kappa-value of 0.65, which is in agreement with the McNemar's test that showed non-significant difference ($P > 0.05$) between proportions of calves classified as having FTPI by the two refractometers. Both refractometers exhibited utility in assessing FTPI in dairy calves; however, digital Brix refractometer showed relatively higher sensitivity, specificity, and accuracy than optical STP refractometer at the optimal cut-offs of 8.3%Brix and 5.5 g/dL, respectively.

7.2 INTRODUCTION

Failure of neonatal calves to absorb colostral IgG is termed failure of transfer of passive immunity (FTPI) (Stott et al., 1979; Sangild, 2003). Calves have FTPI if the serum IgG concentration is <1,000 mg/dL (Weaver et al., 2000; Godden, 2008). FTPI has been associated with reduced growth rates, increased morbidity and mortality, increased risk of being culled, and decreased first-lactation milk production (Robison et al., 1988; Tyler et al., 1999; Virtala et al., 1999; Faber et al., 2005). Consequently, FTPI has effects on the survival, health and productivity of dairy heifers (Smith and Foster, 2007). Prevalence of FTPI among US dairy calves in 2007 was 19 to 40% (Dairy, 2007).

Several assays are available for evaluating the adequacy of transfer of passive immunity in neonatal calves on dairy farms and monitoring FTPI is an important part of preventive herd health protocols (Tyler et al., 1996; Parish et al., 1997; Weaver et al.,

2000). Radial immunodiffusion (RID) is a direct measurement of IgG concentration in calves and, although this test is the industry gold standard (McBeath et al., 1971), it is laboratory-based, relatively expensive, requires a minimum of 18 to 24 hours to obtain the results and, thus, is not practical for routine, on-farm monitoring of adequacy of transfer of passive immunity. Refractometry has been used for many years (McBeath et al., 1971), and other assays, such as the zinc sulfite turbidity test, sodium sulfate turbidity test, serum γ-glutamyl transferase activity, whole blood glutaraldehyde coagulation test, and ELISA have been described as having varying degrees of accuracy for predicting IgG concentrations in dairy calves (Tyler et al., 1996; Parish et al., 1997; Weaver et al., 2000).

Refractometers, either digital Brix or optical refractometer, are an effective on-farm tool to evaluate colostrum quality (Bielmann et al., 2010; Morrill et al., 2012) or determine FTPI in calves (Calloway et al., 2002; Moore et al., 2009), because immunoglobulins are the greatest constituent of total protein in neonatal calf blood besides albumin (McBeath et al., 1971; Tyler et al., 1996; Vandeputte et al., 2011). The relationship between IgG and STP has been confirmed in many studies (Weaver et al., 2000; Godden, 2008). Using an STP concentration of ≤5.0 or ≤5.2 g/dL most accurately predicted FTPI by optical refractometer (Naylor and Kronfeld, 1977; Calloway et al., 2002), while for digital Brix refractometer, a Brix percentage of <7.8%Brix may be used to identify FTPI in dairy calves (Morrill et al., 2013). The objectives of the present study were to determine the sensitivity, specificity and accuracy of both digital and optical refractometers for assessing success of transfer of passive immunity in dairy calves, and to evaluate the agreement between two types of refractometers.

7.3 MATERIALS AND METHODS

7.3.1 Serum samples

Calves (n = 203) from five commercial dairy farms in Prince Edward Island and one farm in Nova Scotia were enrolled in the study between June and October, 2013. Animals eligible for inclusion in this study were Holstein calves from 1 to 11 days of age that appeared physically healthy and were not visibly dehydrated. Whole blood was collected from calves by jugular venipuncture using a 20-gauge, 1-inch hypodermic needle (BD Vacutainer Precision Glide, Becton Dickinson Co., Franklin Lakes, NJ), into a sterile, plastic Vacutainer tube without anticoagulant (BD Vacutainer, Becton Dickinson and Co.). Samples were transported in a cooler to Maritime Quality Milk Laboratory, University of Prince Edward Island (UPEI). Serum was separated by centrifugation at 1,500 g for 10 min at 20 – 24°C within 5 hours of collection. Three aliquots of serum were collected and stored at -80°C. Serum from Nova Scotia samples was separated by centrifugation at 1,500 g for 10 min at a local veterinary clinic and then frozen at -20°C until transported to UPEI. In the end, 3 samples were missing and thus not available for analysis (n = 200). This study was approved by the University of Prince Edward Island Animal Care Committee and performed according to the guidelines of the Canadian Council on Animal Care (Care, Canadian Council On Animal, 2009).

7.3.2 RID analysis

A commercial RID assay (Bovine IgG RID Kit, Triple J Farms; Bellingham, WA) was used as the reference method for determining IgG serum concentrations. Serum

samples were allowed to thaw at room temperature (20 – 24°C) and then vortexed for 10 seconds. Subsequently, the RID assay was performed according to manufacturer's instructions, using 5µL of undiluted serum sample in each well. The same manufacturer's standards (the same lot) were used on all RID assays. Diameters of precipitated rings were measured after 18 – 24 hours of incubation at room temperature, using a handheld calliper. Each of the samples and assay standards were tested in replicates of five. The averages of the five replicates of the assay standards were used to build a calibration curve that was subsequently used to determine IgG concentrations for the serum samples. The final IgG concentration for each sample was determined by calculating the average of the five replicates. Serum samples with IgG concentrations greater than the manufacturer's stated performance range for the assay (>3,000 mg/dL) were diluted (50:50) with deionized sterile water and retested.

7.3.3 Digital Brix and optical refractometers analysis

Serum samples were thawed at room temperature and vortexed for 10 seconds, then tested by the digital Brix refractometer (Atago Co. Ltd; WA, USA) and the optical STP hand-held refractometer (Westover RHC-200ATC handheld STP refractometer, Woodinville, Washington, USA) to assess FTPI. For the digital Brix refractometer, approximately 250 µL of serum were used, and then the Brix score of the liquid was determined by shining a light through the sample in the prism, measuring the index of refraction, and representing the reading (%Brix) on a digital scale. For the optical STP refractometer, approximate 100 µL of serum were placed on the prism and the sample cover was lowered. The refractometer was then held up to a light source, and the STP

(g/dL) value was read at the line between the light and dark areas that appeared on the scale. Digital and optical refractometers were cleaned and calibrated with distilled water at the start of each analysis. The Brix scores were determined first by the optical refractometer to avoid any bias in the results by the technician.

7.3.4 Statistical analysis

Descriptive statistics for the results of RID, digital Brix, and optical STP refractometers were calculated. Results from the digital (%Brix) and optical STP (g/dL) refractometers were plotted against each other and against the measured IgG concentration from RID in mg/dL. From these plots, correlation coefficients (*r*) were determined. Epidemiological diagnostic test characteristics (sensitivity, specificity, predictive values and accuracy) were calculated to evaluate clinical applicability of digital Brix and optical STP refractometers for diagnosis of FTPI, using previously recommended cut-off values (Godden, 2008; Morrill et al., 2013; Deelen et al., 2014) and IgG concentrations of <1,000 mg/dL (measured by RID test) as FTPI positive cases (Tyler et al., 1996; Godden, 2008). Sensitivity was defined as the proportion of calves with serum IgG concentration <1,000 mg/dL that were test-positive. Specificity was defined as the proportion of calves with serum IgG concentration ≥1,000 mg/dL that were test-negative. A receiver operating characteristic curve (ROC) was created to plot the true positive rate against the false positive rate for both the digital and optical refractometers using (roctab command in stata). The computed Se, Sp and accuracy for each of the possible cut-off values were tabulated and the best cut-off value was defined as the one give optimum combination of Se, Sp and accuracy. The area under the curve (AUC) in

the ROC plot, with a 95% confidence interval (CI), was calculated. AUC, a commonly used index of the overall ability of a test to discriminate a target condition, was used to compare the performances of each of the refractometers (Hanley and McNeil, 1982). The level of agreement between results of digital Brix and optical STP refractometers were assessed using McNemar's test (Lachenbruch and Lynch, 1998) for paired data to check for bias, followed by calculation of the kappa statistic.

7.4 RESULTS

7.4.1 Descriptive analysis

The frequency distribution of the RID IgG concentrations was skewed to the right (Figure 7.1A) and ranged from 133 to 5,995 mg/dL. The mean and median of the RID IgG concentrations were 1,765 mg/dL (SD ±1,039) and 1,594 mg/dL, respectively. The distribution of Brix and STP concentrations appeared approximately normally distributed (Figure 7.1B and 7.1C). The mean and median of Brix concentrations as measured by digital Brix refractometer were 8.8%Brix (SD ±1.03) and 8.7%Brix, respectively, with a range of 5.9 to 12.9%Brix. The mean and median of STP concentrations as measured by optical refractometer were 6.0 g/dL (SD ±1.0) and 5.8 g/dL, respectively, with a range of 4.2 to 10.6 g/dL. Fifty-five out of 200 samples had IgG concentrations based on RID below a cut-off value of 1,000 mg/dL for FTPI positive cases, generating a true FTPI prevalence of 27.5%.

7.4.2 Correlation coefficients

Correlation between each refractometer and the RID assay was determined using correlation plots of 200 serum samples. The RID IgG concentration was positively correlated with Brix concentration measured by digital refractometer (r = 0.79, Figure 7.2A) and with STP measured by optical refractometer (r = 0.74, Figure 7.2B). Similarly, the correlation between digital Brix and optical refractometer results was 0.73 (Figure 7.2C).

7.4.3 Diagnostic test characteristics

The test characteristics of the digital Brix and optical STP refractometers were determined for assessment of FTPI (serum IgG <1,000 mg/dL). The Se, Sp, PPV, NPV, and accuracy for digital Brix and optical STP refractometer at previously recommended cut-points are shown in (Table 7.1). A receiver operating characteristic curve was created to plot the true positive rate against the false positive rate for both digital Brix and optical STP refractometers (Figure 7.3). The cut-point of 8.3%Brix on digital refractometer yielded the best combination of Se (85.5%; 95% CI: 75.5 – 94.7%), Sp (82.8%; 95% CI: 78.7 – 90.8%) and accuracy (83.5%). Similarly, the best combination of Se (80%; 95% CI: 67 – 89.6%), Sp (80.7%; 95% CI: 73.3 – 86.8%) and accuracy (80.5%) for optical STP refractometer was achieved at 5.5 g/dL STP. The AUC of digital Brix and optical STP refractometers were 0.89 (95% CI: 0.85 – 0.94) and 0.88 (95% CI: 0.82 – 0.93), respectively.

7.4.4 Agreement between tests

The agreement between results of the two refractometers and RID assay for assessment of FTPI is presented in Table 7.2. The overall percent of agreement between results of digital Brix and optical STP refractometers was 85%, with a corresponding kappa-value of 0.65. The McNemar's test for the sensitivity and specificity comparison, showed no significant difference (P = 0.77 and 0.17, respectively), between the proportion of calves classified as having FTPI by the two refractometers.

7.5 DISCUSSION

Fifty-five out of 200 samples had IgG concentrations <1,000 mg/dL based on RID, generating a true FTPI prevalence of 27.5%, which is consistent with the FTPI prevalence previously reported in the literature (Tyler et al., 1996; Dairy, 2007; Trotz-Williams et al., 2008; Windeyer et al., 2014). However, the average values for serum IgG, %Brix, and STP concentrations determined in this study are slightly lower and the ranges wider than those found in a recently published paper (Deelen et al., 2014).

The correlation coefficients of the two refractometers compared to the RID determinations were similar. The Brix concentration measured by digital refractometer was positively correlated with serum IgG (r = 0.79; Figure 7.2A). However, higher correlation between digital refractometer and RID assay (r = 0.87) was reported, when Caprylic acid fractionation used to improve estimates of refractometer (Morrill et al., 2013). STP determined by optical refractometer was also positively correlated with serum IgG concentration (r = 0.74, Figure 7.2B). Although, similar correlation between STP

and IgG as measured by RID (r = 0.72) have been reported (McBeath et al., 1971), others have observed correlation coefficients from 0.67 to 0.93 between STP and IgG concentrations (Villarroel et al., 2013; Deelen et al., 2014). This could be attributed to the use of digital refractometer to determine the STP in those studies. In the current study, STP determine by optical refractometer was positively correlated with %Brix (r = 0.73; Figure 7.2C). Perfect correlation (r = 1.0) between STP and %Brix was reported in a recent study (Deelen et al., 2014). However, in that study they used the same digital refractometer to determine both the STP and Brix concentrations, which might explain the perfect correlation (Deelen et al., 2014).

For this study, the diagnostic test characteristics were established for both digital Brix and optical STP refractometers. These calculations provided an opportunity to identify utility and differences between both types of refractometers in assessment of FTPI in dairy calves. The diagnostic test characteristics of both refractometers were determined at previously recommended cut-off values (Table 7.1). For example, the Se and Sp of digital refractometer at 8.4%Brix (Deelen et al., 2014) were 89.1 and 76.6%, respectively. The sensitivity and specificity of optical refractometer at 5.2 g/dL (Godden, 2008) were 69.1 and 89%, respectively. The test characteristics determined were different at various cut-offs, which is related to a change of apparent prevalence of FTPI in the study population that altered the proportion of calves correctly identified (Calloway et al., 2002). Se for detecting FTPI increased from 32.7% at 7.8%Brix to 89.1% at 8.4%Brix and from 25.5% at 5.0 g/dL to 87.3% at 5.7 g/dL (Table 7.1). In this study, the Se (85.5%) and Sp (82.8%) of digital Brix refractometer at a cut-off value 8.3%Brix were very close to the Se (88.9%) and Sp (88.9%) reported in a recent study, which used a cut-

off value 8.4%Brix (Calloway et al., 2002). Similarly, the Se (80.0%) and Sp (80.7%) of the optical STP refractometer at a cut-off value 5.5 g/dL were very similar to the Se (80%) and Sp (80%) previously reported with a cut-off value of 5.2 g/dL (Calloway et al., 2002). The differences in the optimal refractometer cut-off values between studies might be attributed to instrument variation between refractometers used in these different studies.

The level of agreement between results of the two refractometers and RID IgG was high (Table 7.2). Similarly, the digital Brix refractometer showed high agreement (85%) with optical refractometer. This indicates that the two refractometers agree on the classification of calves with and without FTPI. Further, the McNemar's test showed a non-significant difference ($P > 0.05$) between proportions of calves classified as having FTPI by the two refractometers, indicating that the two refractometers performed similarly for FTPI assessment in calves.

7.6 CONCLUSIONS

Both the digital Brix and optical STP refractometers show good potential for being useful management tools to be included in the calf health monitoring program on dairy operations. The results from this study suggest that the appropriate cut-off values for digital Brix and optical STP refractometers are 8.3%Brix and 5.5 g/dL, respectively, to assess FTPI in dairy calves. The two refractometers performed similarly in detection of FTPI.

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Table 7.1. Diagnostic test characteristics for the digital Brix and optical refractometers for assessing failure of transfer of passive immunity (FTPI) using previously recommended cut-off values compared with immunoglobulin G (IgG) (<1,000 mg/dL) determined by radial immunodiffusion (RID) assay.

Refractometer	Test characteristics					
	Cut-off value	Sensitivity (%)	Specificity (%)	PPV[a] (%)	NPV[b] (%)	Accuracy (%)
Digital Brix values (%Brix)	7.8	32.7	94.5	69.2	78.7	77.5
	8.0	47.3	93.1	72.2	82.3	80.5
	8.2	76.4	85.5	66.7	90.5	83
	8.4	89.1	76.6	59	94.9	80
Optical STP values (g/dL)	5	25.5	98.6	87.5	77.7	78.5
	5.2	69.1	89	70.4	88.4	83.5
	5.5	80	80.7	61.1	91.4	80.5
	5.7	87.3	69	51.6	93.5	74

[a] Positive predictive value
[b] Negative predictive value

Table 7.2. Level of agreement between results of refractometers and radial immunodiffusion (RID) assay for assessing of failure of transfer of passive immunity (FTPI) in 200 dairy calves.

Refractometers	Cut-off value	Agreement	kappa	P-value
Digital Brix	8.3%Brix	0.83	0.59	0.0001
Optical STP	5.5 g/dL	0.81	0.55	0.0001
Digital Vs. Optical	-	0.85	0.65	0.0001

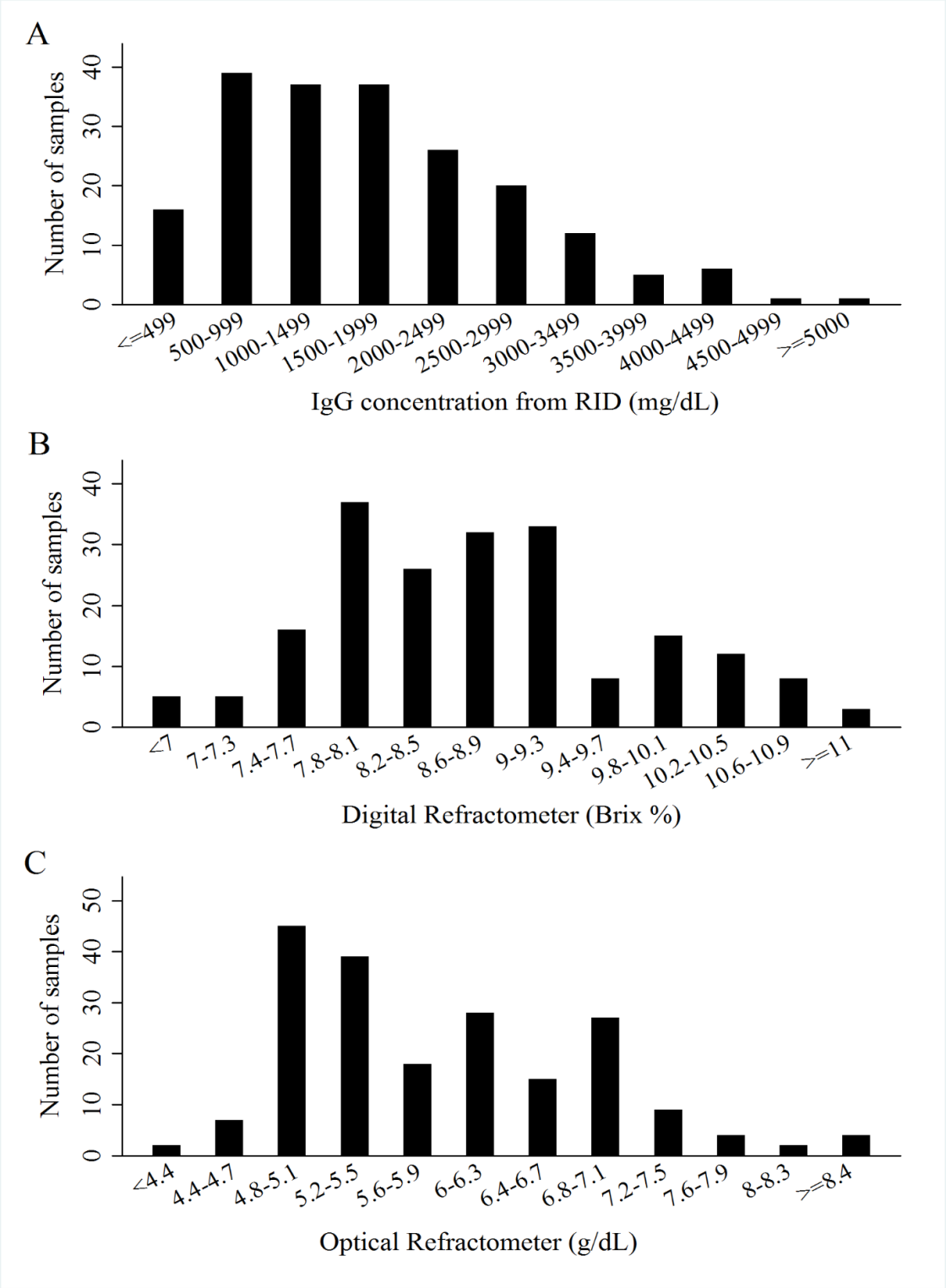

Figure 7.1. Frequency distributions of (A) serum immunoglobulin G (IgG) concentration measured by radial immunodiffusion (RID) assay, (B) percentage Brix (%Brix) determined by digital Brix refractometer, and (C) serum total protein (STP) determined by optical refractometer for 200 Holstein dairy calves.

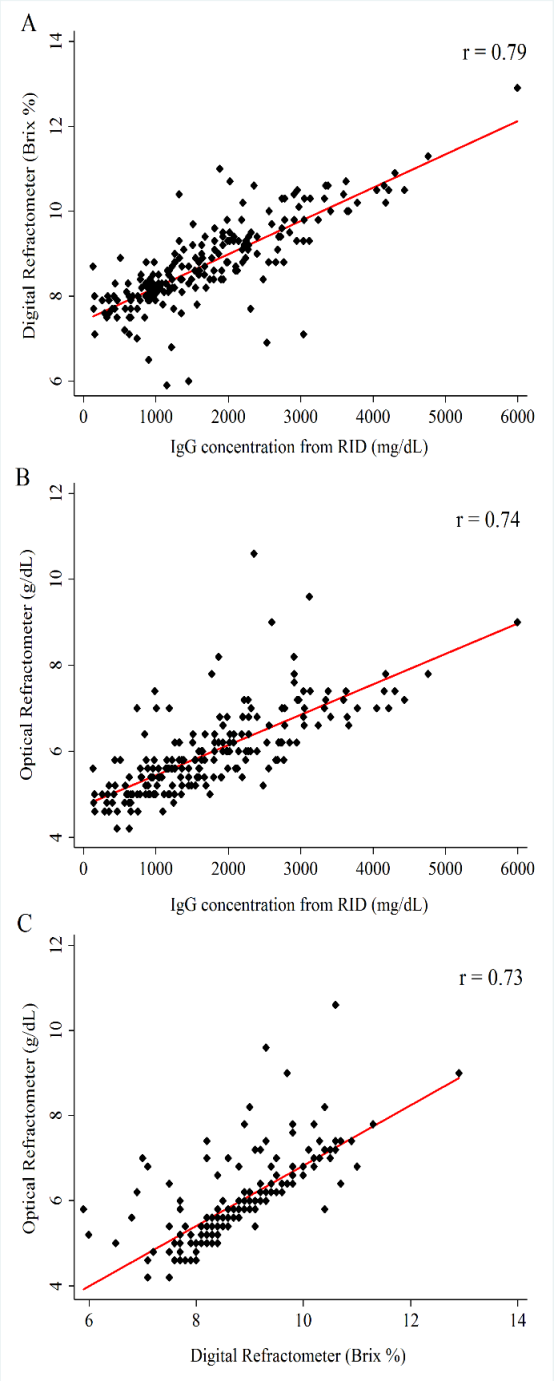

Figure 7.2. Scatter plots comparing (A) serum immunoglobulin G (IgG) concentration obtained by radial immunodiffusion (RID) assay with percentage Brix (%Brix), determined by digital refractometer (r = 0.79); (B) serum immunoglobulin G (IgG) concentration obtained by radial immunodiffusion (RID) assay with serum total protein (STP), determined by optical refractometer (r = 0.74); (C) Percentage Brix (%Brix), determined by digital refractometer with STP obtained by optical refractometer (r = 0.73) for 200 Holstein dairy calves.

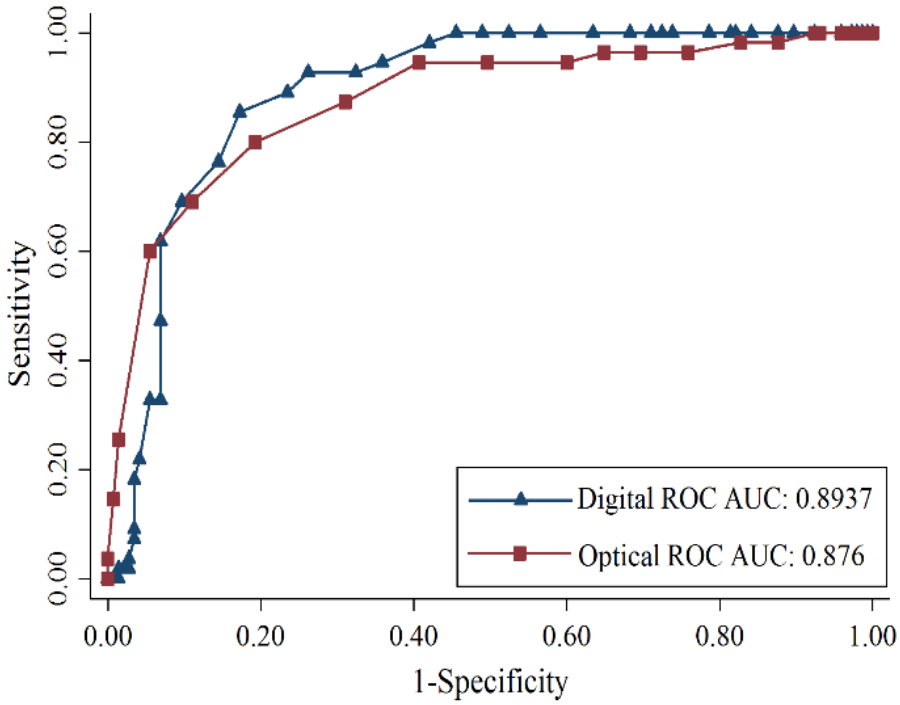

Figure 7.3. Receiver operating characteristic (ROC) curve, the best combination of sensitivity and specificity for digital Brix and optical STP refractometers obtained at a cut-off value 8.3%Brix and 5.5 g/dL, respectively.

CHAPTER 8

EVALUATION OF DIGITAL AND OPTICAL BRIX REFRACTOMETERS FOR ASSESSING COLOSTRUM QUALITY IN DAIRY CATTLE

This chapter is submitted as: I. Elsohaby, J.T. McClure, and G.P. Keefe. Evaluation of digital and optical brix refractometers for assessing colostrum quality in dairy cattle. Australian Veterinary Journal (Under review).

8.1 ABSTRACT

The objectives of this study were to evaluate the digital and optical Brix refractometers for measurement of colostral IgG concentration and the level of agreement between the two refractometers for assessment of colostrum quality. Colostrum samples (n = 245) were collected from Holstein dairy cows on 7 commercial dairy farms. Colostral IgG concentrations were measured by the reference method radial immunodiffusion (RID) assay. The Brix scores were determined by both the digital and optical refractometers. The mean RID IgG concentration was 47.6 g/L (SD ± 28.3), with a range from 4.2 to 144.2 g/L. The mean of %Brix concentration determined by the digital refractometer was 22.7%Brix (SD ± 4.2) with a range from 10.2 to 29.8%Brix, whereas, for the optical refractometer was 23.1%Brix (SD ± 3.9) with a range from 7.4 – 30%Brix.

The Spearman correlation between RID IgG concentration and Brix scores determined by the digital and optical refractometers were 0.59 and 0.57, respectively. The correlation between Brix scores from the digital and optical refractometers was 0.96. Test characteristics of the digital and optical refractometers for assessment of good quality colostrum (IgG ≥ 50 g/L) using previously recommended cut-off points were calculated. The Se, Sp and accuracy of the digital refractometer, at a cut-point of 22%Brix, were 88.4, 54.7 and 67.8%, respectively. Similarly, the Se, Sp and accuracy of the optical refractometer, at a cut-point of 22%Brix, were 90.5, 49.3 and 65.3%, respectively. The best combination of Se (75.8%), Sp (69.3%) and accuracy (71.8%) for digital refractometer was at 23.7%Brix, while for the optical refractometer the best combination of Se (71.6%), Sp (68%) and accuracy (69.4%) was at 24.2%Brix.

The overall percent of agreement between results of the digital and optical refractometers was 93%, with a corresponding kappa-value of 0.85, which is in agreement with the McNemar's test that showed non-significant difference between proportions of colostrum samples classified as poor and good quality by the two refractometers. Both refractometers exhibited moderate utility in assessing colostrum quality. There was strong agreement between the two refractometers. However, the optimal cut-offs for the digital (23.7%Brix) and optical (24.2%Brix) refractometers were slightly higher than previously recommended cut-offs.

8.2 INTRODUCTION

Colostrum is a critical source of immunoprotection and nutrition for newborn calves (Bielmann et al., 2010). Ingestion of good quality colostrum during the first 24 h of life is essential for the future health and performance of dairy calves (Rauprich et al., 2000). Insufficient ingestion and/or absorption of colostral IgG results in failure of transfer of passive immunity (FTPI) (Calloway et al., 2002; Godden, 2008). Calves with FTPI are more susceptible to infectious diseases and have higher morbidity and mortality rates (Robison et al., 1988; Donovan et al., 1998; Virtala et al., 1999). Thus, it is important to assess colostrum quality before feeding to calves. However, only 13% of US dairy producers routinely evaluate colostrum quality as part of their replacement management (Dairy, 2007).

Several methods have been developed to evaluate colostrum quality by measuring the colostral IgG concentration either directly or indirectly, but few of them are applicable to farm or field conditions. The radial immunodiffusion (RID) assay is

regarded the most accurate reference method for directly measuring bovine colostral IgG content (McBeath et al., 1971; Oyeniyi and Hunter, 1978; Fleenor and Stott, 1980). However, this assay is a laboratory based method, requires 18 to 24 h to obtain results, has high cost, lacks automation, and utilizes reagents with a short shelf-life (Riley et al., 2007; Bielmann et al., 2010). Thus, the RID assay is not practical for timely routine on-farm monitoring of colostrum quality before feeding or storage.

The colostrometer and refractometer are the most common indirect methods for evaluating colostrum quality on-farm. Colostrometers measure the specific gravity of colostrum and provide an estimate of relative quality, not precise IgG concentrations (Fleenor and Stott, 1980; Pritchett et al., 1991). Results from the colostrometer are affected by colostrum temperature and total solids content other than IgG (Mechor et al., 1992; Morin et al., 2001). Moreover, it is fragile and needs to be carefully cleaned before use (Bielmann et al., 2010; Bartier et al., 2015). Only 4% of farms in Quebec have been reported to use the colostrometer consistently (Vasseur et al., 2010).

Brix refractometers, either digital or optical, are used to estimate colostral IgG levels and therefore assess colostrum quality (Bielmann et al., 2010; Bartier et al., 2015; Morrill et al., 2015). Refractometery estimates the total protein concentration in a solution (Chavatte et al., 1998). Previous studies reported a strong correlation between Brix scores and IgG concentrations (Chigerwe et al., 2008; Bielmann et al., 2010; Bartier et al., 2015). Unlike the colostrometer, refractometry is not sensitive to temperature (Bielmann et al., 2010; Bartier et al., 2015) and less fragile. Thus, they have been promoted by the dairy industries as an effective on-farm tool to estimate the colostral IgG and to assess colostrum quality (Thornhill et al., 2015). The objectives of this study were

to determine the sensitivity, specificity, and accuracy of both digital and optical Brix refractometers for assessing colostrum quality in dairy cows, and to evaluate the agreement between the two refractometers.

8.3 MATERIALS AND METHODS

8.3.1 Colostrum samples collection

Colostrum samples (n = 251) were collected from first milking after calving from Holstein dairy cows on 7 commercial dairy farms in Prince Edward Island, Canada. The first 168 samples were collected between June and October 2013, while the remaining 83 were collected between May and August 2014. For each cow, a 50 mL colostrum sample was collected in a vial labelled with the cow identification and date of collection, and then stored on the farm at -20°C until transportation to the Maritime Quality Milk Laboratory, University of Prince Edward Island (UPEI), where the samples were stored at -80°C. Animal care and ethics approvals were obtained from the Animal Care Committee at UPEI, and all procedures followed the guidelines of the Canadian Council on Animal Care (CCAC) guidelines (CCAC, 2009).

8.3.2 Colostrum sample analysis

8.3.2.1 Radial immunodiffusion assay

A commercial RID assay (Bovine IgG RID Kit; Triple J Farms; Bellingham, WA) was used as the reference method for measuring colostral IgG concentrations. Colostrum samples were thawed at room temperature (20°C – 24°C) and vortexed for 10 s. Thawed samples were diluted (1:3) with deionized sterile water and mixed by vortexing at a

maximum of 2,700 rpm for 10 s. After dilution, The RID assay was performed according to manufacturer's instructions, using 5 μL of diluted colostrum in each well, tested alongside the manufacturer's standard. RID plates were incubated at room temperature for 18 - 24 h, and the precipitating ring diameter surrounding the well was measured using a hand-held calliper. Colostrum samples with IgG concentration greater than the manufacture's stated performance range for the assay (>3,000 mg/dL) were diluted (1:5) with deionized sterile water and retested. To account for plate-to-plate variation, the same IgG standard (same lot) was used on all RID assays, and each of the assay standards and colostrum samples was tested in replicates of five. The average of the assay standards was used to build a calibration curve that was used to determine the IgG concentration for each colostrum samples. The final IgG concentration for each sample was determined by calculating the average of the 5 replicates. The results were considered acceptable if the coefficient of determination of the calibration curve was equal to or greater than 0.97.

8.3.2.2 Digital and optical Brix refractometers

Colostrum samples were thawed at room temperature and vortexed for 10 seconds, then tested using a digital Brix refractometer (PAL-1, Atago Co. Ltd., Bellevue, WA) with scale from 0 to 52%Brix and an optical Brix refractometer (model 300001; SPER Scientific, Scottsdale, AZ) with scale from 0 to 32%Brix. Both the digital and optical Brix refractometers measure the refractive index of liquid on a Brix scale (%Brix). For the digital Brix refractometer, approximate 250 μL of colostrum were used, and then the Brix score of the liquid was determined by transmitting light through the sample in the prism and recording the reading on a digital scale. For the optical Brix

refractometer, approximate 50 μL of colostrum were placed on the prism and the sample cover was lowered. The refractometer was then held up to a light source and the Brix score was read at the blue line between the light and dark area that appeared on the scale.

Digital and optical refractometers were cleaned and calibrated with distilled water at the start of each analysis. The Brix scores were determined first by the optical refractometer to avoid any bias in the results by the technician. Colostrum samples with Brix score greater than the upper limit of the optical refractometer (32%Brix) were excluded from further analysis (n = 6).

8.3.3 Statistical analysis

Descriptive statistics were used to compare Brix scores determined by the digital and optical refractometers with the IgG concentrations obtained from the reference RID assay. The digital and optical refractometers Brix scores (%Brix) were plotted against each other and against the RID IgG concentration (g/L). The correlation between both refractometers and the colostral RID IgG concentration was determined using the Spearman correlation coefficient. The concordance of the digital and optical refractometers was determined using Lin's concordance correlation coefficient (Lin, 1989).

Epidemiological diagnostic test characteristics (sensitivity, specificity, predictive values and accuracy) were calculated to evaluate the performance of digital and optical refractometers in differentiation between poor and good quality colostrum using previously recommended cut-off values and ≥ 50 g/L of RID IgG concentration as the cut-point for good quality colostrum. Six cut-off values at 1%Brix intervals from 20 –

25%Brix were considered. Sensitivity was defined as the proportion of good quality samples that are correctly identified as such, and specificity was defined as the proportion of poor quality samples that are correctly identified as such. Accuracy was defined as the proportion of colostrum samples that were correctly classified. Predictive values were calculated using the prevalence of samples with RID IgG concentration of greater than or less than 50 g/L in the study population and from previously reported studies and a %Brix below or above the recommended cut-points.

A receiver operating characteristic curve (ROC) was created to plot the true positive rate against the false positive rate for both the digital and optical refractometers. The computed Se, Sp, and accuracy for each of the possible cut-off values were tabulated and the best cut-off value was defined as the one give optimum combination of Se, Sp, and accuracy. The area under the curve (AUC) in the ROC plot, with a 95% confidence interval (CI), was calculated. AUC, a commonly used index of the overall ability of a test to discriminate a target condition, was used to compare the performances of each of the refractometers. The level of agreement between results of digital and optical refractometers were assessed using Bland-Altman plot and McNemar's test (Lachenbruch and Lynch, 1998) for paired data to check for bias, followed by calculation of the kappa statistic. These calculations were performed with Stata version 13.0 statistical software (Stata Corporation, 2013).

8.4 RESULTS

8.4.1 Descriptive statistics

The RID IgG concentrations displayed a right skewed distribution (Figure 8.1) and ranged from 4.2 to 144.2 g/L. The mean and median of the RID IgG concentrations were 47.6 g/L (SD ± 28.3) and 39.8 g/L, respectively. Only 39% (95 of 245) of samples had IgG concentrations above the cut-off value of 50 g/L. The distribution of the digital and optical refractometers Brix scores were skewed to the left (Figure 8.2). The Brix scores determined by the digital refractometer had a mean and median of 22.7%Brix (SD ± 4.2) and 23.5%Brix, respectively, with a range of 10.2 – 29.8%Brix. The mean and median of Brix scores measured by the optical refractometer were 23.1%Brix (SD ± 3.9) and 24%Brix, respectively, with a range of 7.4 – 30%Brix.

8.4.2 Correlation coefficients

The correlation between each refractometer and the RID assay was examined using correlation plots of the 245 colostrum samples (Figure 8.3). The correlation between the RID-measured IgG concentrations and Brix scores determined by the digital and optical refractometers were 0.59 and 0.57, respectively. The correlation between Brix scores from the digital and optical refractometers was 0.96. In addition, it was found that the concordance correlation coefficient between both refractometers was 0.96 (Figure 8.4).

8.4.3 Diagnostic test characteristics and predictive values

The digital and optical refractometers were analyzed for test characteristics against the reference RID for assessment of colostrum quality. A positive sample (good

quality colostrum) was defined as containing RID IgG concentration ≥ 50 g/L. The Se, Sp, PPV, NPV and accuracy for the digital and optical refractometers at previously recommended cut-off values are shown in Table 8.1. A ROC was created to plot the true positive rate against the false positive rate for the digital and optical refractometers (Figure 8.5). The AUC of the digital and optical refractometers were 0.75 (95% CI: 0.69 - 0.81) and 0.74 (95% CI: 0.68 - 0.80), respectively. Subsequently, the best combination of Se (75.8%; 95% CI: 65.9 - 84%), Sp (69.3%; 95% CI: 61.3 - 76.6%), PPV (61%; 95% CI: 51.6 - 69.9%), NPV (81.9%, 95% CI: 74.1 - 88.2%) and accuracy (71.8%) were determined for the digital refractometer at a cut-point of 23.7%Brix. The best combination of Se (71.6%; 95% CI: 61.4 - 80.4%), Sp (68%, 95% CI: 59.9 - 75.4%), PPV (58.6%; 95% CI: 49.1 - 67.7%), NPV (79.1%, 95% CI: 71 - 85.7%) and accuracy (69.4%) were achieved for the optical refractometer at a cut-point of 24.2%Brix. The PPV and NPV of the two refractometers were relatively low. Figure 8.6 shows the predictive ability of the digital and optical Brix refractometers at various prevalences of good quality colostrum (IgG ≥ 50 g/L), including recently reported prevalence values from herds in the United States (Morrill et al., 2012). The agreement between results of the two refractometers and RID assay for assessment of bovine colostrum quality is presented in Table 8.2.

8.4.4 Agreement between refractometers

The overall percentage of agreement between Brix scores of the digital and optical refractometers was 93%, with a corresponding kappa value of 0.85. The agreement between two refractometers was further assessed using the Bland-Altman plot

(Figure 8.7), which revealed that the mean value of the difference between Brix scores provided by the two refractometers was 0.4%Brix, indicating no obvious systematic bias between two instruments. The 95% confidence interval range was -1.7 to 2.5%Brix. The McNemar's test for the sensitivity and specificity comparison, showed no significant difference (P = 0.22 and P = 0.77, respectively), between the proportion of colostrum samples classified as poor and good quality by the two refractometers.

8.5 DISCUSSION

In current study, the average colostral IgG concentration (47.29 g/L) was lower than recently published averages which ranged from 63.7 to 94.9 g/L (Bielmann et al., 2010; Quigley et al., 2013; Bartier et al., 2015). However, the range (4.2 – 144.2 g/L; Figure 8.1) of colostrum IgG concentrations as measured by RID was close to that reported by Morrill et al., (2015; 12.8 – 154 g/L), Quigley et al., (2013; 7.1 – 159 g/L) and higher than the range reported by Bartier et al., (2015; 8.3 – 128.6 g/L). Moreover, in this study the percentage (61%) of colostrum samples with RID IgG concentration <50 g/L was higher than the percentage of poor colostrum samples previously reported in these studies (Bielmann et al., 2010; Morrill et al., 2015; Bartier et al., 2015). Consequently, the mean of Brix scores determined by digital (22.7 %Brix) and optical (23.1 %Brix) refractometers were lower than previously reported means for digital (26.4 %Brix) and optical (26.1 %Brix) refractometers (Bielmann et al., 2010). There are several factors that may explain the difference in the distribution of IgG concentration values in the present study and previous studies. Sample size may have played a role, as in this study, only 245 cows from 7 herds were enrolled, whereas, 569 cows from thirteen

farms, 827 cows from 67 farms and 288 cows from three herds were used in Bartier et al., (2015), Morrill et al., (2012) and Bielmann et al., (2010) studies, respectively. The larger number of farms included the greater variability in the environmental, management and nutritional practices that are known to affect colostrum quality (Godden, 2008). The time of colostrum collection (Hostetler et al., 2003; Chigerwe et al., 2008), genetic variation (Baumrucker et al., 2010) and number of freezing-thawing cycles of colostrum samples before testing (Farrant, 1980; Morrill et al., 2015) have all been reported to contribute to the difference in IgG distribution. Furthermore, in this study sample collection was done by cooperating farmers. While, farmers were instructed to collect first feeding samples, it is possible that second or later feeding colostrum samples were included.

The correlation coefficients between Brix scores determined by the two refractometers and IgG concentration measured by the reference RID assay (Figure 8.3) were not as high as values previously reported in studies to evaluate use of digital and optical refractometers for assessing bovine colostrum quality (Bielmann et al., 2010; Morrill et al., 2015; Bartier et al., 2015). Variations in the correlation coefficients in these studies could be due to the differences in the non-IgG contents in the colostrum. The Brix refractometers measures IgG concentrations indirectly, through measuring total dissolved solids in colostrum, which are affected by dry period length (Rastani et al., 2005), vaccination status of the dam (Hodgins and Shewen, 1996), and season of calving (Morin et al., 2001). The concordance correlation coefficient between Brix scores obtained from two refractometers (0.96; Figure 8.4) was higher than concordance correlation (0.87) recently reported for evaluation the use of digital and optical refractometers to assess passive immunity transfer in serum of dairy calves (Thornhill et al., 2015).

The diagnostic test characteristics of the digital and optical refractometers were established. These calculations provided an opportunity to identify utility and differences between the two refractometers in the assessment of colostrum quality. Appropriate %Brix cut-points need to be determined to ensure that good quality colostrum is fed to newborn calves. For both the digital and optical refractometers, the previously published cut-points for good quality colostrum ranged from 18 to 23%Brix (Bielmann et al., 2010; Morrill et al., 2012; Bartier et al., 2015). For this study, the optimal cut-points for the digital and optical refractometers were at 23.7%Brix and 24.2%Brix, respectively. These cut-points achieved the greatest combination of Se, Sp and accuracy (Table 8.1). It has been suggested that the sensitivity of a colostrum quality test is more important than specificity (Morrill et al., 2015). However maximizing the specificity of the refractometer would lead to a decrease in number of samples inaccurately classified as good quality colostrum (>50 g/L), increasing the PPV and reducing feeding and storage of colostrum with insufficient IgG (<50 g/L). The diagnostic test characteristics determined in this study were close to those reported by Bartier et al., (2015) and Quigley et al., (2013) for the digital refractometer and different than that reported by Bielmann et al., (2010) for both the digital and optical refractometers. This difference could be attributed to the q instrument variation between refractometers used in these different studies. Figure 8.6 illustrates the predictive values of the digital and optical Brix refractometers with varying prevalences of good quality colostrum. The digital Brix refractometer showed relatively higher PPV and NPV than the optical Brix refractometer at good quality colostrum prevalence of 39% reported in this study and the recently published prevalence from herds in the United States (Morrill et al., 2012).

The digital and optical refractometers showed moderate agreement with RID IgG (Table 8.2). However, the digital refractometer showed almost perfect agreement with the optical refractometer based on the kappa analysis. The lack of systemic bias (Bland-Altman plot) and lack of significant difference demonstrable using the McNemar's test indicates that the digital and optical refractometers performed similarly for assessing of colostrum quality. Similar results were reported for the use of digital and optical refractometers in assessment of colostrum quality (Bielmann et al., 2010) and passive immunity transfer in dairy calves (Thornhill et al., 2015; Elsohaby et al., 2015).

8.6 CONCLUSIONS

Brix refractometers, either digital or optical show potential for being an effective management tools to be included in colostrum monitoring program. This study demonstrates that the cut-points of the digital and optical refractometers for optimizing Se, Sp and accuracy of colostrum measurement are 23.7%Brix and 24.2%Brix, respectively. The most appropriate cut-point depends on the relative importance placed on false negative versus false positive results. The two refractometers performed similarly in assessment of colostrum quality, but demonstrated only moderate agreement with the RID reference method.

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Table 8.1. Diagnostic test characteristics for the digital and optical Brix refractometers for assessing of colostrum quality using previously recommended cut-off points compared with IgG (≥50 g/L) determined by radial immunodiffusion (RID) assay

Refractometers	Test charactristics[1]					
	Cut-off (%Brix)	Se (%)	Sp (%)	PPV (%)	NPV (%)	Accuracy (%)
Digital	20	96.8 (91–99.3)	37.3 (29.6–45.6)	49.5 (42.1–56.9)	94.9 (85.9–98.9)	60.4
	21	94.7 (88.1–98.3)	47.3 (39.1–55.6)	53.3 (45.4–61)	93.4 (85.3–97.8)	65.7
	22	88.4 (80.2–94.1)	54.7 (46.3–62.8)	55.3 (47–63.3)	88.2 (79.8–93.9)	67.8
	23	83.2 (74.1–90.1)	63.3 (55.1–71)	59 (50.1–67.4)	85.6 (77.6–91.5)	71
	24	67.4 (57–76.6)	73.3 (65.5–80.2)	61.5 (51.5–70.9)	78 (70.3–84.5)	71
	25	52.6 (42.1–63)	78.7 (71.2–84.9)	61 (49.6–71.6)	72.4 (64.9–79.1)	68.6
Optical	20	98.9 (94.3–100)	30.7 (23.4–38.7)	47.5 (40.4–54.7)	97.9 (88.7–99.9)	57.1
	21	96.8 (91–99.3)	41.3 (33.4–49.7)	51.1 (43.6–58.6)	95.4 (87.1–99)	62.9
	22	90.5 (82.8–95.6)	49.3 (41.1–57.6)	53.1 (45.1–61)	89.2 (80.4–94.9)	65.3
	23	86.3 (77.7–92.5)	56.7 (48.3–64.7)	55.8 (47.4–64)	86.7 (78.4–92.7)	68.2
	24	76.8 (67.1–84.9)	62.7 (54.4–70.4)	56.6 (47.6–65.3)	81 (72.7–87.7)	68.2
	25	60 (49.4–69.9)	74 (66.2–80.8)	59.4 (48.9–69.3)	74.5 (66.7–81.3)	68.6

[1]Se = sensitivity; Sp = specificity; PPV = positive predictive value; NPV = negative predictive value; Accuracy = number of correctly classified colostrum samples.

Table 8.2. Level of agreement between the digital, optical Brix refractometers and radial immunodiffusion (RID) assay for evaluating colostrum quality in 245 colostrum samples

Refractometers	Cut-off (%Brix)	Agreement	Kappa
Digital vs. RID	23.7	0.72	0.43
Optical vs. RID	24.2	0.69	0.38
Digital vs. Optical	-	0.93	0.85

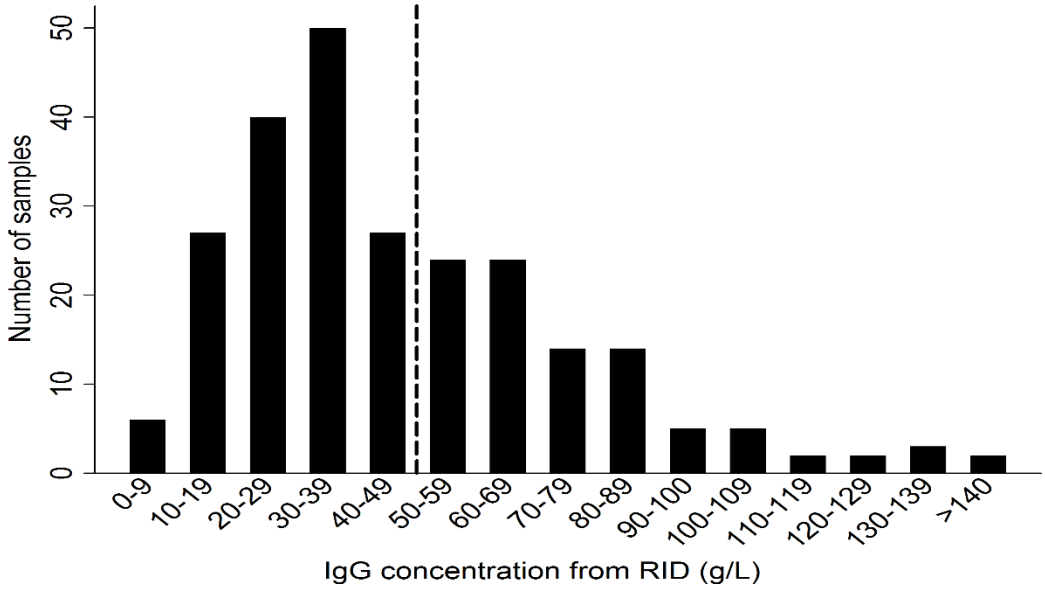

Figure 8.1. Distribution of colostral IgG concentrations measured by radial immunodiffusion (RID) assay for 245 colostrum samples. The dashed line represents the cut-off point between poor and good quality colostrum (IgG = 50 g/L).

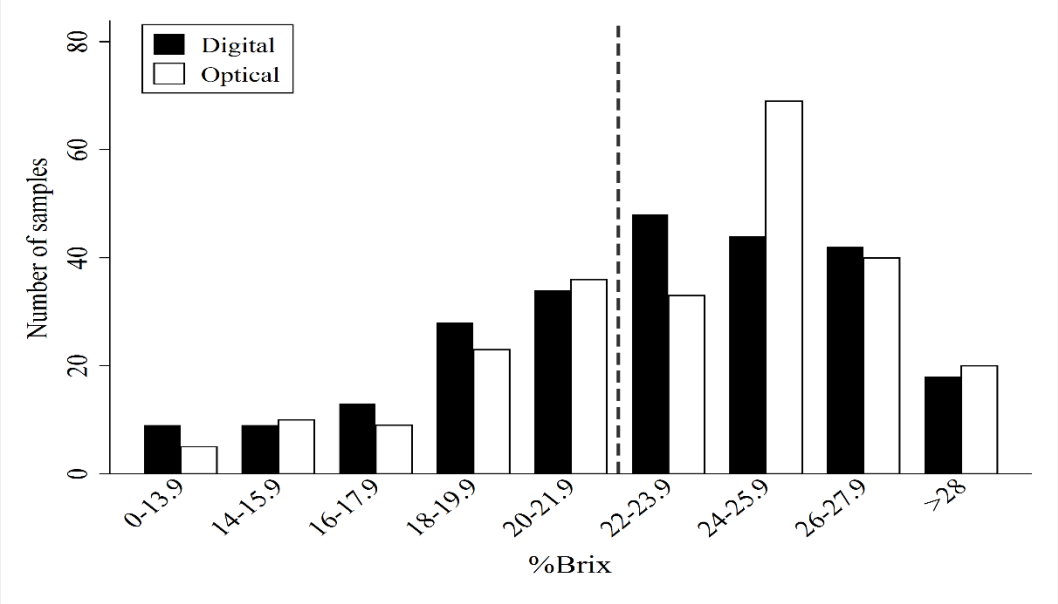

Figure 8.2. Distribution of Brix scores (%Brix) measured by the digital and optical refractometers for 245 colostrum samples. The dashed line represents a previously recommended cut-off point between poor and good quality colostrum (Brix score = 22%Brix).

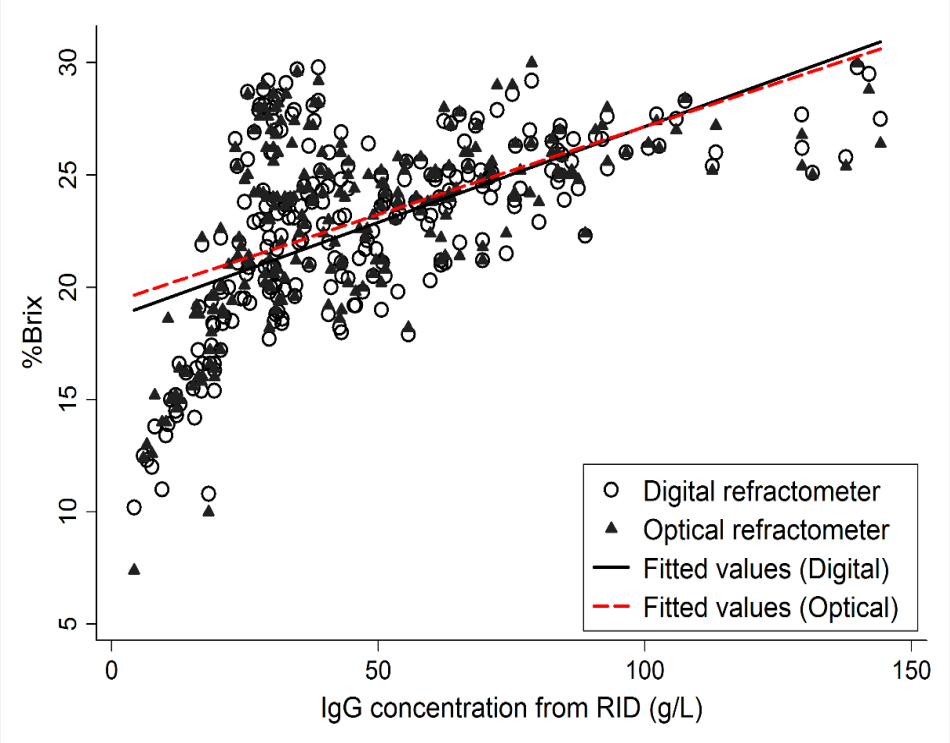

Figure 8.3. Scatter plot of colostral IgG concentrations obtained by radial immunodiffusion (RID) assay and Brix scores obtained by both the digital and optical refractometers for 245 colostrum samples.

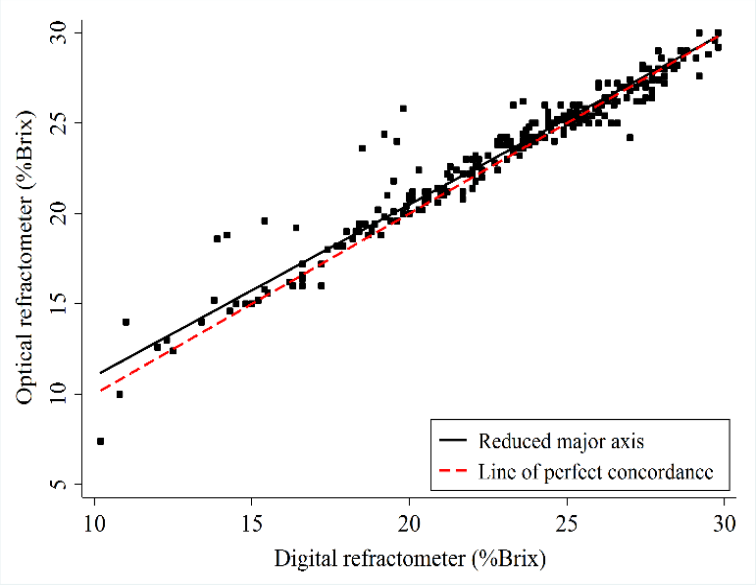

Figure 8.4. Lin's concordance correlation plot for Brix scores obtained from the digital and optical refractometers for 245 colostrum samples.

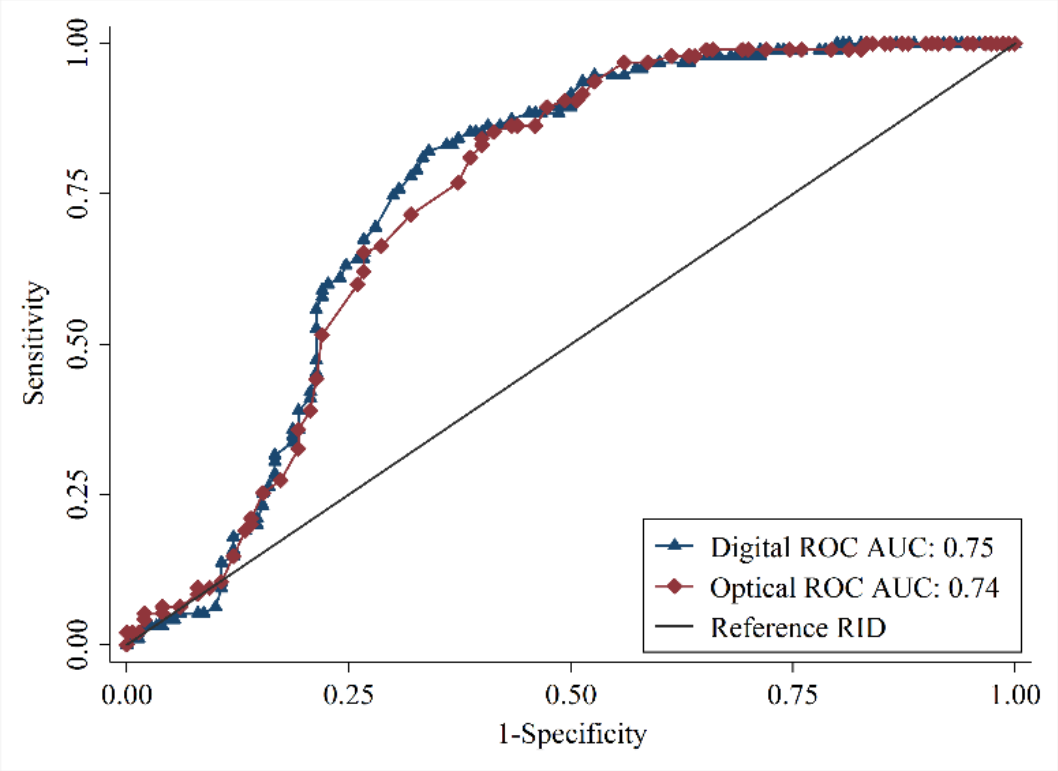

Figure 8.5. Receiver operating characteristic (ROC) curve, the best combination of sensitivity and specificity for the digital and optical refractometers obtained at a cut-off point 23.7%Brix and 24.2%Brix, respectively.

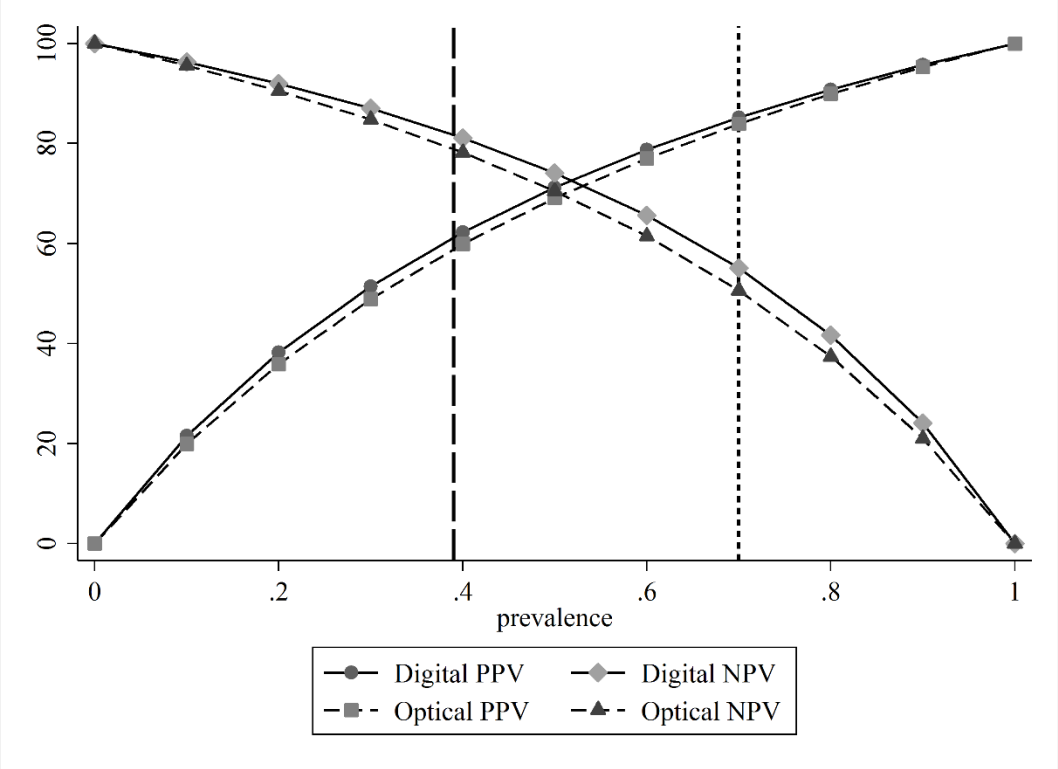

Figure 8.6. Positive predictive values (PPV) and negative predictive values (NPV) for the digital and optical Brix refractometers obtained at a cut-off point 23.7%Brix and 24.2%Brix, respectively. The long and short dashed lines represent the good quality colostrum prevalence reported in this study (0.39) and from herds in the United States (0.70) (Morrill et al., 2012), respectively.

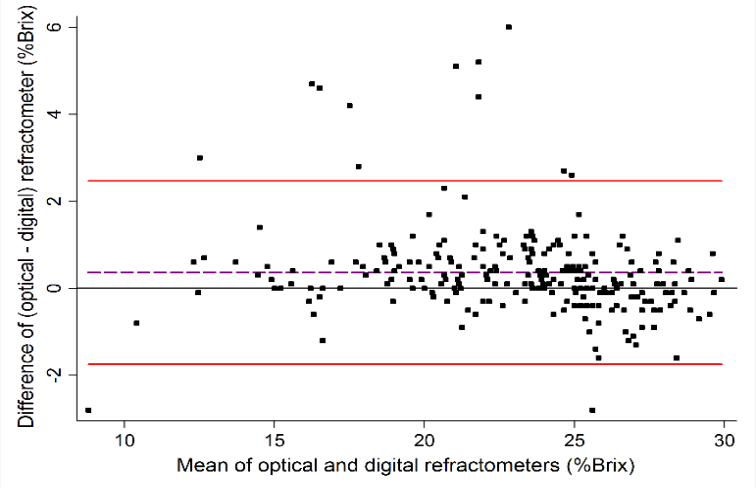

Figure 8.7. Bland-Altman plot of the difference in the Brix scores measured by the digital and optical refractometers for 245 colostrum samples. The solid lines represent the 95% confidence limits of agreement (–1.7 to 2.5%Brix). The horizontal dashed line represents the mean difference between the optical and digital refractometers (0.4%Brix).

CHAPTER 9. SUMMARY AND CONCLUDING REMARKS

9.1 INTRODUCTION

Despite progress in veterinary medicine, failure of transfer of passive immunity (FTPI) still occurs in North America with prevalence ranging between 11 and 31% in beef calves (Perino, 1997) and between 19 and 40% in dairy calves (Nocek et al., 1984; Dairy, 2007). Calves with FTPI are more susceptible for early neonatal calf losses associated with gastroenteritis, pneumonia and septicemia. The effects of FTPI extend beyond the neonatal period, affecting long-term productivity, decreasing milk yield and increasing culling rates during first lactation (DeNise et al., 1989; Heinrichs and Heinrichs, 2011).

Level of colostrum and calf health management practices have a profound effect on success of transfer of passive immunity. Proper management during the first few days of calf life can markedly reduce morbidity and mortality, whereas, improper management lead to economic losses from increased cost of veterinary intervention, death losses, reduced growth and suboptimal reproductive performance (Quigley, 2004).

Several methods have been used to measure serum and colostrum IgG concentrations and to assess colostrum and calf health management in dairy farms. The radial immunodiffusion assay (RID) is acknowledged as the reference test for the direct quantification of bovine serum and colostrum IgG concentrations (McBeath et al., 1971), but it has significant drawbacks as it takes 18-24 hours to obtain the results, utilizes

reagents and is expensive (Liu et al., 2007; Riley et al., 2007). Therefore, this dissertation focuses on two main objectives:

1- Development and validation of a novel method (IR-based assay) for rapid and accurate quantification of bovine IgG concentrations, for detection of FTPI in dairy calves, and for assessing colostrum quality of dairy cows.
2- Validation of novel and current on-farm methods (ZAPvet bovine IgG test and refractometers) as rapid, indirect on-farm tools for detection of FTPI in dairy calves and for assessing of colostrum quality of dairy cows in comparison with the reference RID assay.

9.2 GENERAL THESIS OVERVIEW

This thesis developed and validated a quantitative assay for bovine serum and colostral IgG, based upon use of IR spectroscopy in combination with a partial least squares regression method. The hypothesis was that IR spectroscopy, in combination with multivariate data analysis, could be used for quantification of bovine IgG in sera and colostrum. The initial step was to develop PLS model for quantification of bovine serum and colostral IgG, then validation of the model against the reference test, then determine the test characteristics and predictive values of the IR-assay when used for detection of FTPI in dairy calves and colostrum quality in dairy cows. Two PLS models, one for transmission IR spectroscopy and second for ATR-IR spectroscopy were built for measuring serum IgG concentration. Comparison between diagnostic performances of the two models for detection of FTPI in dairy calves were performed using a new sample

data set rather than the samples used in model building. In addition, this thesis evaluated the diagnostic characteristics of a novel on-farm method (ZAPvet Bovine IgG test) for detection of FTPI in dairy calves and validated current on-farm tools (digital and optical refractometers when used for diagnosis of FTPI in dairy calves and colostrum quality of dairy cows.

9.3 SUMMARY OF THE MAIN FINDING

9.3.1 Measurement of IgG concentration: Novel methods

9.3.1.1 Infrared spectroscopy

In Chapter 2 and 3, a total of 250 pre and post-colostrum serum samples were collected from dairy calves and were used to develop an algorithm for measuring serum IgG concentration using transmission-IR and ATR-IR spectroscopy in combination with PLS regression. The serum samples were divided into two sets: combined and test sets. The combined set was used for building a calibration model, while the test set was used to assess predictive ability of the calibration model. Both transmission-IR and ATR-IR based assays were developed, to quantify serum IgG concentration and detection of FTPI in dairy calves. The IgG concentrations measured by the IR-based assays showed excellent Pearson and concordance correlation coefficients with those measured by the RID assay. However, the level of disagreement between RID and IR based assays at high IgG concentration is greater than that at low concentrations. The difference between RID and IR assays is acceptable. The RID assays provided by different manufacturers could

give results that differ by as much as 40%. This is thought to be due to the differences in standards used to determine calibration curves (Ameri and Wilkerson, 2008).

The transmission-IR based assay (RPD = 2.9 and RER = 9.7) showed better utility for the prediction of serum IgG concentrations than the ATR-IR based assay (RPD = 2.7 and RER = 9.1) (Williams and Sobering, 1996). Furthermore, the precision of transmission IR-based assay was higher than that of ATR-IR based assay, and both assays were lower than that of the reference RID assay. However, it lies within the acceptable range of the quality control standards of the US Food and Drug Administration Agency (US Department of Health and Human Services, 2001). The IR-based assays showed good to excellent sensitivity and specificity, when used for diagnosis of FTPI in dairy calves. According to the reference RID assay, the prevalence of FTPI (IgG <1000 mg/dL) was 51%, while the prevalence of FTPI determined by the transmission and ATR-IR based assays were 51% and 47%, respectively. Although the transmission-IR based assay showed higher potential for measurement of serum IgG concentration than the ATR-IR based assay, it is still predominantly a laboratory based analytic method. However, the ATR-IR assay is more appropriate for use under field conditions as it is rugged and portable (Sun, 2009; Smith, 2011).

In this study, the effects of different spectral data pre-processing techniques on model performance and predictive accuracy were investigated. We found that, the spectral derivation significantly improved the performance and accuracy of the transmission-IR based assay. However, spectral smoothing (without spectral derivation) significantly improved the ATR-IR based assay performance and accuracy.

In Chapter 4, the feasibility of the transmission-IR and ATR-IR based assays for measuring serum IgG concentration and diagnosis of FTPI in a new data set were examined. The serum IgG concentration of 203 calves from 6 dairy herds were measured by the reference RID, transmission-IR and ATR-IR assays. The IgG concentration measured by the reference RID was highly correlated with the IgG concentration predicted by the transmission-IR (r = 0.94) and ATR-IR (r = 0.92) based assay. In this study, the prevalence of FTPI as determined by the reference RID was 27.5%, while the prevalence of FTPI determined by the transmission-IR and ATR-IR based assays were 26.5% and 28%, respectively. The level of agreement between results from the two IR-based assays and RID assay for detection of FTPI was high. Furthermore, the two IR based assays demonstrates a high level of agreement on classification of calves with and without FTPI. The transmission-IR based assay misclassified 12 samples as false positives and false negatives, while the ATR-IR based assay misclassified 8 samples as false positives. The transmission-IR based assay showed relatively minor higher specificity, PPV and precision than the ATR-IR based assay.

In Chapter 5, a total of 251 colostrum samples were collected from dairy cows originating from 7 dairy herds and were used to develop a transmission-IR based assay for quantification of colostral IgG concentration and for assessment of colostrum quality. Methods similar to those used in chapter 2 were employed to develop the IR assay. The colostrum samples were tested by the reference RID assay and transmission-IR spectroscopy. The developed IR assay showed utility for prediction of colostral IgG (RPD = 2.3 and RER = 11), which was highly correlated (r = 0.91) with the IgG concentration measured by RID assay. Although, the transmission-IR based assay for

measuring colostral IgG concentration showed lower precision than that of the reference RID assay, it showed high applicability for assessing poor quality colostrum with overall accuracy of 90%, sensitivity of 88% and specificity of 94%. The IR-based assay misclassified only 24 samples out of 250 colostrum samples as false positives (8 samples) and false negatives (16 samples).

9.3.1.2 ZAPvet Bovine IgG test

The focus for Chapter 6 was to evaluate the utility and test characteristics of an initial version of calf-side test for diagnosis of FTPI in dairy calves. A total 202 serum samples, collected from 6 dairy herds were tested by the ZAPvet test and the reference RID assay. The results of ZAPvet test were interpreted by two observers. According to the RID assay, the prevalence of FTPI was 27%, while the prevalence of FTPI derived from ZAPvet test was 47.5% for observer #1 and 41% for observer #2. Based on the results derived from observer #1, the level of agreement between ZAPvet test and RID assay was relatively high with observed agreement of 82%. The ZAPvet test is relatively sensitive (82%) and would be acceptable as an initial screening test for diagnosis of FTPI in dairy calves, and is more efficient than submitting serum samples to an accredited veterinary diagnostic laboratory for IgG testing. However, the low specificity (65%) of ZAPvet test would result in over prediction of FTPI incidence, which could result in unnecessary interventions for calves without FTPI. The overall percent of agreement between results derived from two observers was 84%, with a kappa value of 0.67. Furthermore, the results from two observers showed no significant difference between proportion of calves with and without FTPI. A photos for finished ZAPvet tests for

samples with different IgG concentration were provided and this makes the interpretation process easier. Consequently, producers will be able to perform the ZAPvet test with minimal training.

9.3.2 Measurement of IgG concentration: Current Methods

9.3.2.1 Digital and optical refractometers

The focus for chapter 7 and 8 was to evaluate the diagnostic performance and difference between digital and optical refractometers for assessing of FTPI in dairy calves and colostrum quality of dairy cows. In chapter 7, a total of 200 serum samples were collected from dairy calves at 6 dairy herds then tested by the reference RID assay, digital Brix refractometer and optical refractometer. The correlation between IgG concentration measured by RID and Brix scores measured by digital Brix refractometers was higher than that with STP measured by optical refractometer. The best combination of sensitivity and specificity for detection of FTPI were achieved at a cut-point of 8.3%Brix and 5.5 g/L for digital Brix and optical refractometers, respectively. Furthermore, the digital and optical refractometers showed moderate agreement with RID assay for assessing FTPI.

In Chapter 8, a total of 251 colostrum samples were collected from 7 dairy farms then tested by the reference RID assay, digital and optical Brix refractometers. Nearly two third of the colostrum samples measured by RID have IgG concentration below 50 g/L (poor quality colostrum). The RID measured IgG concentrations were better correlated with the Brix scores measured by digital Brix refractometer than that measured

by optical Brix refractometer. For assessing colostrum quality, the digital Brix refractometer had the highest combined sensitivity and specificity at a cut-point of 23.7%Brix, which is higher than the reported cut-points in previous studies (Chigerwe et al., 2008; Bielmann et al., 2010; Quigley et al., 2013; Bartier et al., 2015). However, the highest proportion of correctly identified good and poor quality samples by optical Brix refractometer occurred at the 24.7%Brix cut-point. This cut-point is higher than the reported data (Bielmann et al., 2010).

9.4 CONCLUDING REMARKS

This research evaluated the usefulness of infrared spectroscopy in combination with multivariate data analysis for rapid and accurate quantification of IgG in bovine serum and colostrum. The new IR based assays showed potential both for detection of FTPI in dairy calves and for assessing colostrum quality; both assays showed with good to excellent accuracy, sensitivity and specificity when compared to the reference RID assay. The new IR based assay is rapid, accurate, reagent-free and inexpensive and thus, overcome the drawbacks of the RID assay. The results are obtained from IR based assay within 4 to 5 minutes and that allows the identification of calves with FTPI prior to gut closure (Weaver et al., 2000). Transmission-IR based assay showed higher potential for accurately measuring IgG concentration than the ATR-IR based assay. However, the ATR-IR based assay showed higher feasibility for measuring IgG concentration under field conditions, and there is potential to improve accuracy by longer signal averaging.

The preliminary results of ZAPvet Bovine IgG test suggest that it has potential for monitoring FTPI on farms or in veterinary clinics. The ZAPvet test is relatively sensitive and would be acceptable as an initial screening test for diagnosis of FTPI in dairy calves, and is more efficient than submitting serum samples to an accredited veterinary diagnostic laboratory for IgG testing. However, the low specificity of ZAPvet test would result in over prediction of FTPI incidence, which could result in unnecessary interventions for calves with ATPI.

Both the digital and optical refractometers showed a good potential for being a useful on-farm management tools to be included in colostrum and calf health monitoring program on dairy operations. Furthermore, the two refractometers performed similarly for detection of FTPI in dairy calves and for assessing of colostrum quality of dairy cows with a cut-points slightly higher than that reported in recent studies (Chigerwe et al., 2008; Bielmann et al., 2010; Quigley et al., 2013; Bartier et al., 2015).

9.5 FUTURE RESEARCH DIRECTIONS

The present study provided a basis for the potential use of infrared spectroscopy and multivariate data analysis for quantification of serum and colostrum IgG concentration. The IR-based assay was rapid, accurate, reagent free and inexpensive tool that help dairy producers to improve their colostrum and calf health management program. Although, the IgG concentration measured by the IR-based assay showed high correlation with the RID-measured IgG concentration, the predictive ability and precision of the IR-based assay may be improved by using a different combination of spectral-

preprocessing techniques as well as using another source for the RID kits with lower variability (Ameri and Wilkerson, 2008). The transmission-IR based assay is a laboratory based analytic tool. However, the ATR-IR based assay is more appropriate for field use (Sun, 2009; Smith, 2011). Therefore, it would be an important step to ensure the utility of ATR-IR based assay by validating it under farm and veterinary clinic conditions. The transmission-IR assay for measuring colostral IgG concentration showed good correlation with RID assay. However, a study to evaluate its predictive performance in a new data set rather than the one used in the model building is needed. Also, it will be beneficial for dairy producers to build a model for the ATR-IR spectroscopy and this will open the door for assessing colostrum quality on-farm.

Most current diagnostic tests use serum samples for measuring IgG concentration and assessing FTPI in dairy calves but very few dairy farms own or have access to a centrifuge to separate serum (Deelen et al., 2014). For example, only 2.1% of farms have used refractometers for routine calf health monitoring according to the 2007 National Animal Health Monitoring System report (Dairy, 2007). Therefore, it will be beneficial for dairy producers to evaluate the diagnostic performance of ZAPvet Bovine IgG test using whole blood samples and the refractometer and IR-based assay using serum collected without centrifugation from blood tubes allowed to clot.

Heat treatment of colostrum has emerged as a method to reduce calf exposure to pathogens. Several studies have reported no effect of heat treatment on colostrum IgG concentration measured by RID assay (Godden et al., 2006; Elizondo-Salazar et al., 2010). However, a recent study reported that heat treatment affects colostral IgG concentration measured by ELISA (Gelsinger et al., 2015). Therefore, it would be an

important step to study the effect of heat treatment on colostral IgG concentrations measured by IR-based assay and Brix refractometers and, determine whether there should be a different cut-point (%Brix) for heat treated colostrum.

9.6 REFERENCES

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